Immunophenotypic characterization of lymphocytic infiltration in canine melanocytic tumors

Caracterização imunofenotípica da infiltração linfocítica em tumores melanocíticos caninos

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ABSTRACT
Melanocytic tumors correspond to approximately for 4 to 7% of tumors in dogs and up to 7% of malignant tumours. Melanomas generally exhibit aggressive biological behavior. In humans, abundant tumor-infiltrating lymphocytes (TIL) can be considered a good prognostic factor. Research on TIL in canine melanomas is scarce, and to date, there are no studies to verify its association with established prognostic factors. Our study aimed to evaluate the lymphocyte population in canine melanocytic tumors using immunohistochemical markers, and to relate this to pre-established clinicopathological prognostic variables and cell proliferation index (Ki-67). An exploratory cross-sectional study was conducted with 21 cases in 19 dogs, of which 71.5% were melanotic melanomas, 19% were melanocytomas and 9.5% were amelanotic melanomas. Ki-67 expression was elevated in amelanotic melanomas, indicating aggressive tumor behavior. We detected lymphocytes T and B through CD3 and CD20 markers, respectively, and found that 86.7% of the melanotic melanomas were positive for CD3, whereas 73.3% were negative for CD20. TIL showed a strong association with malignant tumors, as well as a correlation with other pre-established prognostic factors, such as necrosis, ulceration, and nuclear atypia. TIL need to be further investigated to verify its inclusion as a prognostic factor for canine melanomas.

Keywords: melanoma, immunohistochemistry, lymphocytic infiltrate tumor, tumor microenvironment, dog.

RESUMO
Os tumores melanocíticos correspondem a aproximadamente 4 a 7% dos tumores em cães e até 7% dos tumores malignos. Os melanomas geralmente exibem um comportamento biológico agressivo. Em humanos, linfócitos infiltrantes tumorais (TIL) podem ser considerados um bom fator prognóstico. As pesquisas sobre TIL em melanomas caninos são escassas e, até o momento, não há estudos que verifiquem sua associação com fatores prognósticos estabelecidos. Nosso estudo teve como objetivo avaliar a população de

linfócitos em tumores melanocíticos caninos por meio de marcadores imunohistoquímicos e relacionar com as variáveis prognósticas clínico-patológicas pré-estabelecidas e índice de proliferação celular (Ki-67). Foi realizado um estudo transversal exploratório com 21 casos em 19 cães, dos quais 71,5% eram melanomas melanóticos, 19% eram melanocitomas e 9,5% eram melanomas amelanóticos. A expressão de Ki-67 estava elevada em melanomas amelanóticos, indicando comportamento agressivo do tumor. Detectamos linfócitos T e B através dos marcadores CD3 e CD20, respectivamente, e constatamos que 86,7% dos melanomas melanóticos eram positivos para CD3, enquanto 73,3% eram negativos para CD20. TIL apresentou forte associação com tumores malignos, bem como correlação com outros fatores prognósticos pré-estabelecidos, como necrose, ulceração e atipia nuclear. TIL precisa ser mais investigado para verificar sua inclusão como um fator prognóstico para melanomas caninos.

Palavras-chave: melanoma, imunohistoquímica, tumor infiltrado linfocítico, microambiente tumoral, cão.

1 INTRODUCTION

Melanocytic tumors correspond to approximately for 4 to 7% of tumors in dogs and up to 7% of malignant tumours (COTCHIN, 1955; LAPRIE et al., 2001). These neoplasms originate from melanocyte proliferation, and generally exhibit aggressive biological behavior (SULAIMON and KITCHELL, 2003; LACROUX et al., 2012). Melanocytic tumors are found in the oral cavity, skin, and the mucocutaneous junctions (SMITH et al., 2002; BERGMAN, 2007; GOLDSCHMIDT and GOLDSCHMIDT, 2017), occurring with greater frequency and similarity in the skin and oral mucosa (GILLARD et al., 2014). Oral melanomas, which are the most common neoplasms of the canine oral cavity (MUNDAY et al., 2017), generally present local invasion, high metastatic potential and poor prognosis (SMEDLEY et al., 2011).

Clinicopathological prognostic factors for canine melanocytic tumors are already established (MILLANTA et al., 2002; SPANGLER and KASS, 2006; SMEDLEY et al., 2011; LACROUX et al., 2012; VASCELLARI et al., 2013; GILLARD et al., 2014; SIMPSON et al., 2014), with Ki-67 used to determine the cell proliferation index, or the relative number of cells actively involved in the cell cycle (SCHOLZEN and GERDES, 2000; MUKARATIRWA, 2005). Its expression is considered a prognostic factor independent of histological grade (SCASE et al., 2006; WEBSTER et al., 2007). Evaluations of mitotic activity are of prognostic value for melanomas, constituting a tool to determine the cell proliferation rate in humans and animals (ROELS et al., 1999; LAPRIE et al., 2001; MILLANTA et al., 2002; BERGIN et al., 2011; SMEDLEY et al., 2011; LACROUX et al., 2012; VASCELLARI et al., 2013; IUSSICH et al., 2016).

To better understand what happens between the tumor and the immune system, tumor-infiltrating lymphocytes (TIL) can be studied and related to tumor features and prognosis (GOODEN et al., 2011). Therefore, TIL are considered an independent prognostic factor in human melanomas, as was shown in recent studies on thin melanomas (CLEMENTE et al., 1996; TAYLOR et al., 2007; BURTON et al., 2011; GOODEN et al., 2011; ANSELMI JÚNIOR et al., 2012; AZIMI et al., 2012; THOMAS et al., 2013). Lymphocyte infiltration is present in all stages of melanoma development. However, the relationship between the degree of TIL and neoplasm prognosis has not yet been well established (ANSELMI JÚNIOR et al., 2012; AZIMI et al., 2012). Different TIL types can be found in a single tumor, with the individual types playing distinct roles in the tumor microenvironment, both inhibiting and promoting the growth of the melanoma (BERNSEN et al., 2004; YAZDI et al., 2006; OBLE et al., 2009; ANSELMI JÚNIOR et al., 2012).

Research on TIL in canine primary melanocytic tumors is scarce and, to date, there are no studies to verify its association with established clinicopathological prognostic factors (HORIUCHI et al., 2010; MARTÍNEZ et al., 2011). This study aimed to analyze the lymphocyte population in canine melanocytic tumors, using immunohistochemical markers to verify whether there is a relationship between TIL, pre-established clinicopathological prognostic variables (location, tumor size, ulceration, predominant cell type, nuclear atypia, number of mitoses, degree of pigmentation, and tumor necrosis), and cell proliferation index, evaluated by Ki-67 expression. Furthermore, the study also aimed to assess the prognostic value of TIL and compare tumor lymphocyte responses between malignant and benign melanocytic tumors.

2 MATERIAL AND METHODS

A cross-sectional exploratory study was carried out to evaluate the lymphocyte population in canine melanocytic tumors using immunohistochemical markers.

2.1 SAMPLE

Cases of primary canine cutaneous and oral melanocytic tumors were reviewed and studied. Tumors were obtained by surgical removal and necropsy, and diagnosed in a veterinary pathology laboratory. The recorded data were garnered from patient charts, and the clinical history (tumor location) and macroscopic aspect (tumor size) were reviewed.

2.2 HISTOPATHOLOGICAL STUDY

Paraffin blocks of all cutaneous and oral canine melanocytic tumors diagnosed were collected. Next, three-micron thick histological sections were cut, stained with the hematoxylin and eosin (HE) method, and analyzed under an optical microscope. Glass slides were viewed, and histomorphological tumor features, such as predominant cell type, ulceration, nuclear atypia, mitotic activity, mitosis count by visualization in 10 high-magnification fields (HMF) (obj. 40×), degree of pigmentation, TIL, and tumor necrosis, were studied in dermal and submucosal components for cutaneous and oral tumors, respectively. The ulceration and tumor necrosis variables were evaluated as "present" or "absent." As for predominant cell type, melanocytic tumors were classified as epithelioid, fusiform, or mixed. Nuclear atypia, degree of pigmentation, and TIL variables were classified according to intensity: 0 for absent, + for mild, ++ for moderate, and +++ for severe. TIL was also classified as peritumoral, intratumoral, or both.

2.3 IMMUNOHISTOCHEMICAL STUDY

After the histopathological study in HE, paraffin blocks were selected and cut into three-micron thick sections for the immunohistochemical study with the streptavidin-biotin-peroxidase complex method. As it is not easy to distinguish the brown pigment formed by the DAB chromogen from the brown granules of melanin pigment, the melanin removal technique was used in cases with a ++ and +++ degree of pigmentation. Subsequently, sections were left in 5% oxalic acid solution for 10 minutes for the more pigmented cases, and for 5 minutes for the less pigmented ones (http://anatpat.unicamp.br/tecnicashistologicas.html#t5). The blockage of endogenous peroxidase activity was conducted by incubating tissue sections in 10% hydrogen peroxide solution (30 vol.) in a tris buffered saline (TBS) (pH 7.6) for 20 minutes. Antigen retrieval was heat-induced and a citrate buffer (pH 6) was used for 40 minutes in a 500W steam cooker. The anti-melan-A (MART-1, A103, Cell Marque), HMB-45 (HMB-45, Cell Marque), S-100 (4C4.9, Cell Marque), Ki-67 (MIB-1, DakoCytomation), and CD20 (L26, Cell Marque) monoclonal antibodies; and the CD3 (Spring) polyclonal antibody were used, diluted in 1:150, 1:100, 1:100, 1:100, 1:200, and 1:500 phosphate buffered saline (PBS), respectively. Slides were incubated in a humidified chamber for 14–16 hours (overnight) at 4°C with the primary antibody.

The sections were then incubated with biotinylated secondary antibody bonded to streptavidin-peroxidase (LSAB+System HRP kit, KO690, DakoCytomation), with each

step lasting 10 minutes. DAB chromogen was used to develop the slides; Harris hematoxylin stain was used to counterstain, and then the slides were mounted using Canada balsam (Pró-Cito). Positive controls were used for each marker: human melanoma for Melan-A, HMB-45 and S-100, lymph nodes for CD3 and CD20, and the palatine tonsil for Ki-67. Subsequently, the slides were analyzed for immunohistochemical features. Markers were classified as negative (-) in the absence of immunostaining, or positive (+) in the presence of complete or incomplete membrane and cytoplasmic immunostaining, of any intensity, for CD3 and CD20, and of cytoplasmic immunostaining for Melan-A, HMB-45 and S-100. Ki-67 expression was classified, qualitatively, as: 0 for negative expression, 1+ for up to 25% nuclear positive cells, 2+ for cells 26-50% nuclear positive, 3+ for cells 51-75% nuclear positive, and 4+ for more than 75% nuclear positive cells.

2.4 STATISTICAL ANALYSIS

Clinicopathological prognostic variables (tumour size, necrosis, microscopic ulceration, nuclear atypia, mitotic index and lymphocytic infiltration) and immunohistochemical (CD3, CD20, and Ki-67) variables were organized in a contingency table. Relative frequencies, absolute frequencies, or both were obtained through descriptive statistics. The chi-square test was used to verify the association among the categorized variables. However, in tables where the expected frequencies were lower than five, the likelihood ratio test was used as a substitute for the chi-square test. The relationship strength between the presence of TIL with histological and immunohistochemical variables was analyzed with the Rank-biserial correlation and Spearman correlation tests. The data were considered significantly different if probability was lower than 5% ($p < 0.05$). The analysis was carried out with the SPSS™ software version 22.0.

3 RESULTS

3.1 SAMPLE CHARACTERIZATION

This study analyzed 21 canine melanocytic tumors obtained from 19 dogs: 19% were melanocytomas (4/21), 71.5% were melanotic melanomas (15/21), and 9.5% were amelanotic melanomas (2/21). Most of the tumors evaluated were in cutaneous tissue (15/21), with a few cases in the oral cavity (6/21).

3.2 CLINICOPATHOLOGICAL PROGNOSTIC VARIABLES CHARACTERIZATION

The size of the tumors studied was not associated with specific types of melanocytic tumors, because 61.1% of tumors were smaller than 3cm (Figure 1A). The histological evaluation of canine melanocytic tumors (Figure 2A-B-C) considered the presence of necrosis and ulceration (Figure 1B-C), which was associated with melanotic melanomas in 42.9% and 57.1% of the cases, respectively. All amelanotic melanomas (2/21) presented both necrosis and ulceration. As for the degree of nuclear atypia, there was a direct association between the absence of nuclear atypia and melanocytomas; in melanomas, on the other hand, the presence of atypia was observed in different degrees (Figure 1D). Most melanomas (12/21) had a mitotic index>3/10 HMF, showing an association between malignancy and higher mitotic index; in contrast, all melanocytomas had a mitotic index <3/10 HMF (Figure 1E).

Figure 1. Clinicopathological prognostic variables associated with melanocytic tumors in dogs: (A) tumor size; (B) necrosis; (C) microscopic ulceration; (D) nuclear atypia; (E) mitotic index. * indicate significant differences (Chi-square, $p<0.05$).

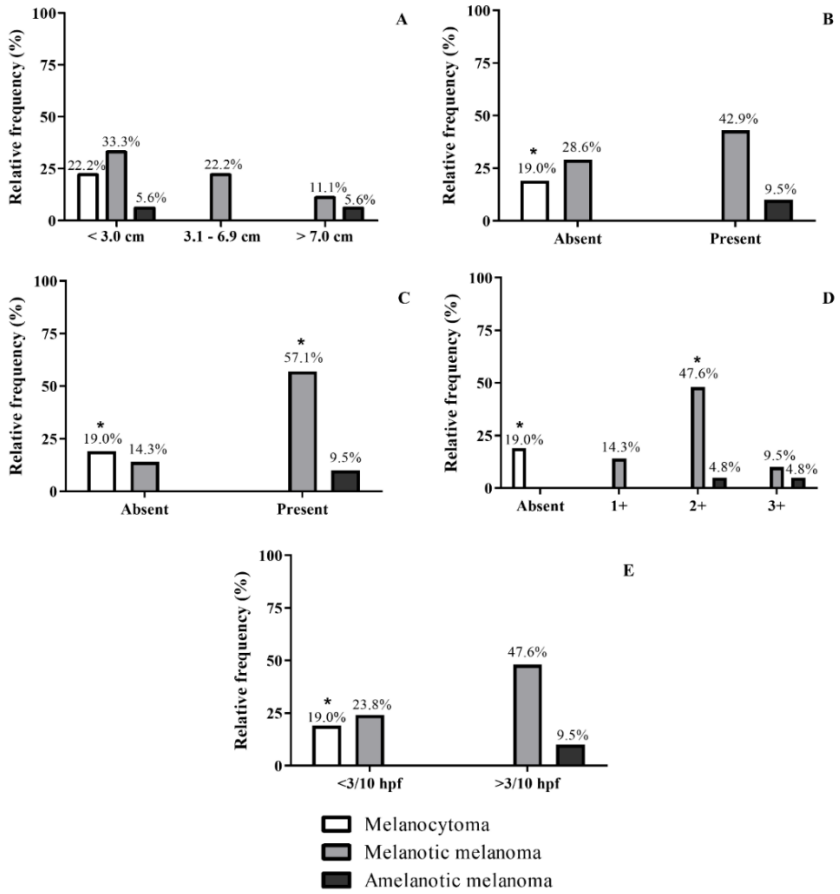
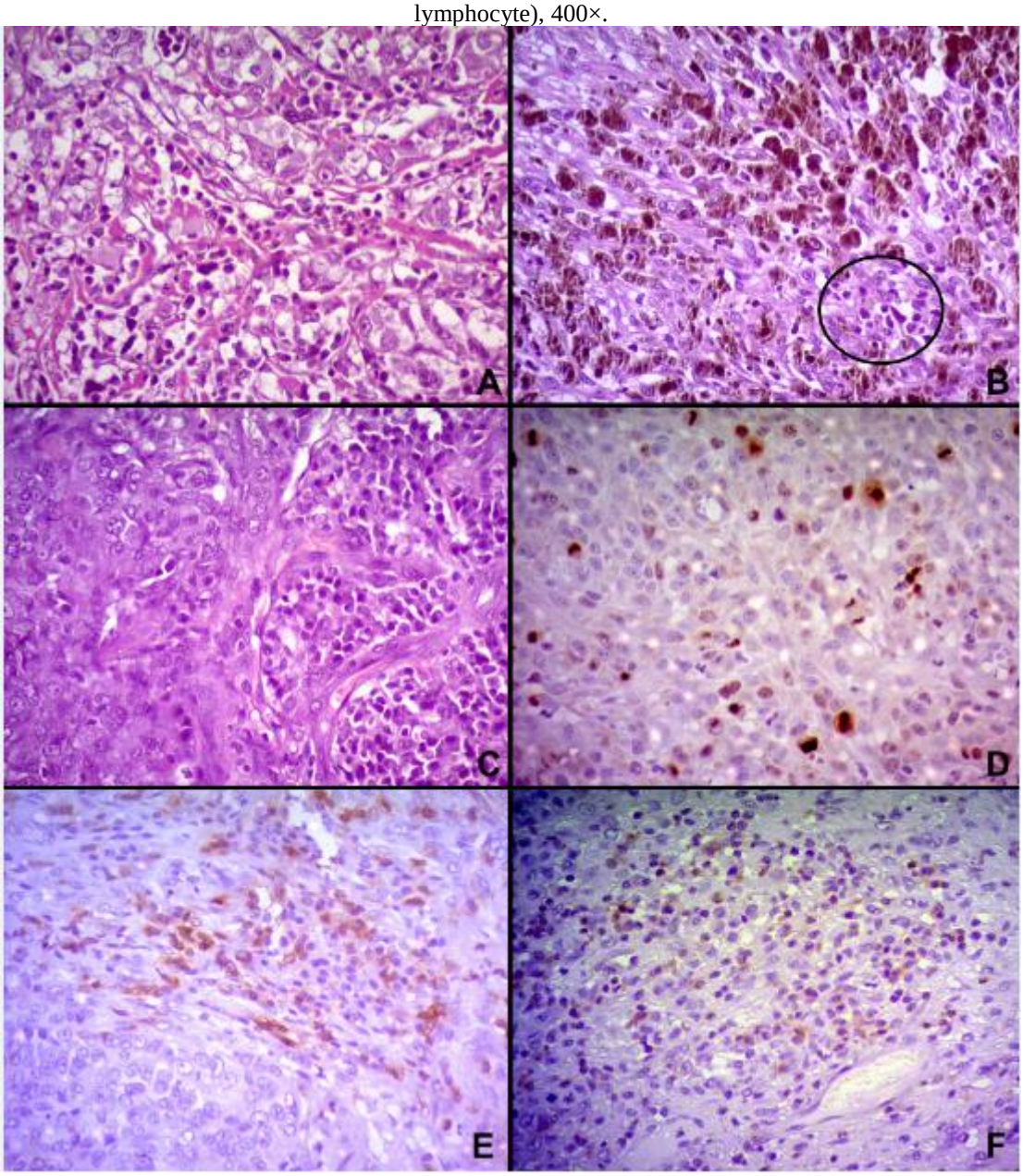


Figure 2. Canine melanocytic tumors. (A) Epithelioid melanotic oral melanoma Intratumoral 3+ TIL HE, 400×. (B) Mixed melanotic cutaneous melanoma Intratumoral 1+ TIL (circle) HE, 400×. (C) Epithelioid amelanotic oral melanoma. Peritumoral 2+ TIL (arrows). HE, 100×. (D) Epithelioid melanotic cutaneous melanoma. IHC, Ki-67 nuclear marking, 400×. (E) Epithelioid amelanotic oral melanoma, peritumoral 2+ TIL. IHC, CD3 membrane and cytoplasmic marking (T lymphocyte), 400×. (F) Mixed melanotic cutaneous melanoma, intratumoral 2+ TIL. IHC, CD3 membrane and cytoplasmic marking (T lymphocyte), 400×.



3.3 IMMUNOHISTOCHEMICAL CHARACTERIZATION

The immunohistochemical markers Melan-A, S-100, and HMB-45 were used to confirm the histogenesis of the tumors studied. Positive markers for Melan-A, S100, and HMB45 were found in 92.5% (20/21), 87.5% (18/21), and 81.0% (17/21) of the cases, respectively, as shown in Table 1.

Table 1. Frequency of immunohistochemical markers in canine melanocytic tumors

	Melan-A N (%)		HMB-45 N (%)		S-100 N (%)	
	Positive	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**
Melanocytoma	4 (19.0%)	0 (0.0%)	4 (19.0%)	0 (0.0%)	4 (19.0%)	0 (0.0%)
Melanotic melanoma	14 (66.7%)	1 (4.8%)	12 (57.1%)	3 (14.2%)	13 (61.9%)	2 (9.5%)
Amelanotic Melanoma	2 (9.5%)	0 (0.0%)	1 (4.8%)	1 (4.8%)	1 (4.8%)	1 (4.8%)
Total	**20 (95.2%)**	**1 (4.8%)**	**17 (81.0%)**	**4 (19.0%)**	**18 (85.7%)**	**3 (14.3%)**

N = absolute frequency; (%) = relative frequency.

The proliferative activity determined by Ki-67 expression in melanocytomas was low (<25%). In contrast, all amelanotic melanomas expressed a high Ki-67 index (>51%). Melanotic melanomas presented a varied distribution, ranging from values lower than 25% to values greater than 26% (Figure 2D and Figure 3A).

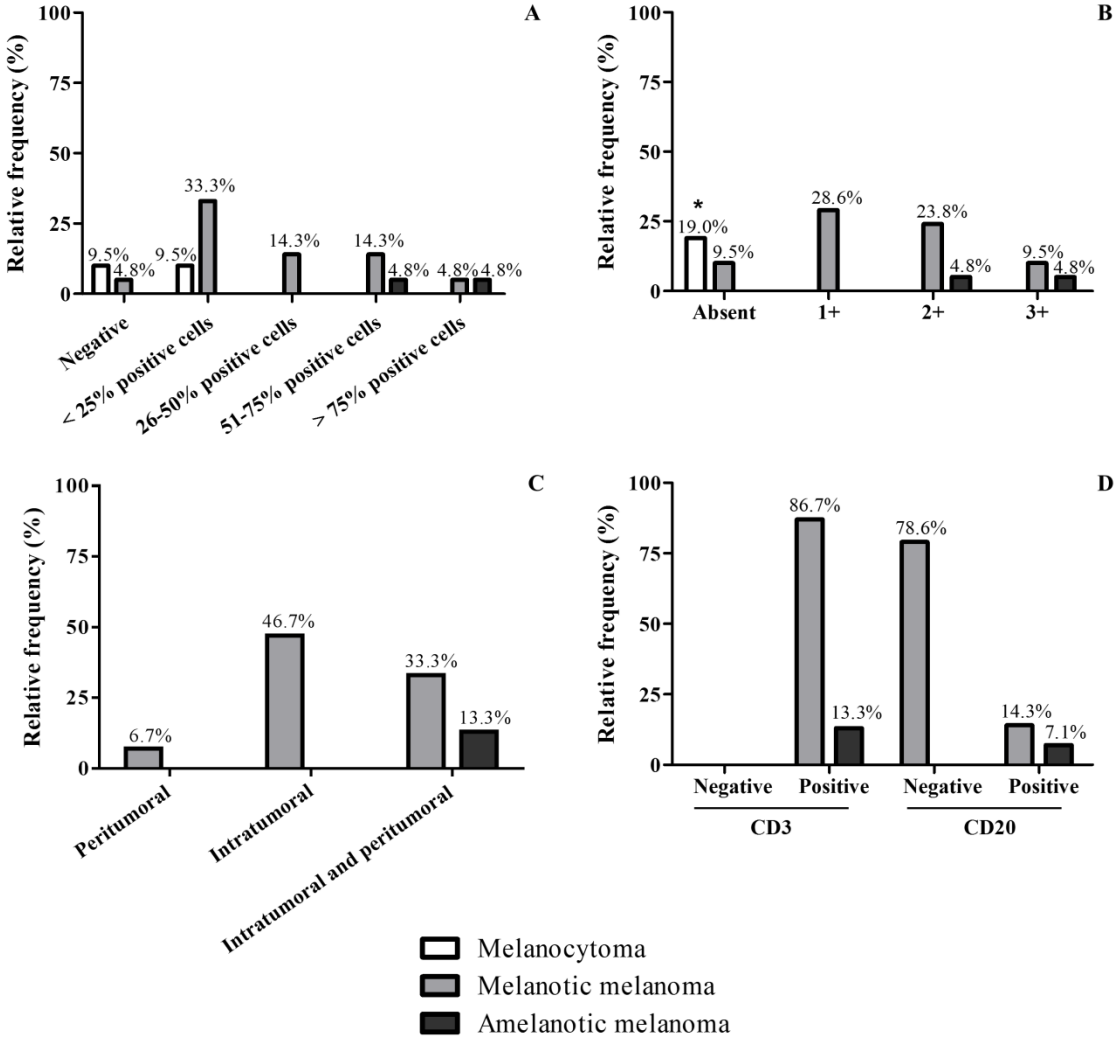
3.4 TUMOR-INFILTRATING LYMPHOCYTES (TIL)

The absence of lymphocytic infiltration was associated with melanocytomas in all cases studied, whereas different degrees of TIL were observed in melanomas, ranging from absent (2/21) to present. In melanomas with present TIL, the majority were with either 1+ (28.6 %) or 2+ (23.8%) infiltration. A few cases were 3+ (9.5%) (Figure 2A). Amelanotic melanomas presented more TILs (2+ and 3+) (Figure 2C) than other melanocytic tumors (Figure 3B).

As regards TIL location in melanomas, there was no association between location and type of melanocytic tumor. Intratumoral infiltration was found in most melanotic melanomas (Figure 2B and F). Intra and peritumoral TIL were found in all amelanotic melanomas (Figure 2C and Figure 3C).

Lymphocyte subtypes in melanotic melanomas were 86.7% (13/15) positive for CD3, whereas 73.3% (11/15) were negative for CD20, showing that most lymphocytes detected were T lymphocytes. Amelanotic melanomas presented a similar distribution of T and B lymphocytes, as shown in Figure 3D.

Figure 3. (A) Ki-67 expression; (B) Presence of lymphocytic infiltration; (C) Infiltration location; (D) CD3 and CD20 expression in canine melanocytic tumors. * indicates significant difference (Chi-square, $p<0.05$).



The relationship strength analysis between TIL and histopathological and immunohistochemical parameters (Table 2) showed a large relationship between the presence of TIL and nuclear atypia ($r_s=0.72$, $p<0.01$), ulceration ($r_{rb}=0.60$, $p<0.01$), and necrosis ($r_{rb}=0.54$, $p=0.01$). Also, a moderate relationship between the presence of TIL and mitotic index ($r_{rb}=0.45$, $p=0.04$), and Ki-67 expression ($r_s=0.40$, $p=0.07$) was observed. Finally, a small relationship was observed between TIL and tumor size ($r_s=0.28$, $p=0.26$).

Table 2. Correlation between lymphocytic infiltrate and histological and immunohistochemical parameters

	Lymphocytic infiltrate	
	r_{rb}	*P*
Microscopic ulceration	0.60	<0.01*
Necrosis	0.54	0.01*
Mitotic index	0.45	0.04*
	r_s	*P*
Tumour size	0.28	0.26
Nuclear atypia	0.72	<0.01*
KI-67 (immunohistochemical)	0.40	0.07

r_{rb} = Rank-biserial correlation coeficiente; r_s = Spearman's correlation coefficient; *p* = statistical significance (*values less than 0.05 indicate a significant difference).

4 DISCUSSION

Our study showed an association between the presence of ulceration and melanomas, similar to what was reported by CAMPAGNE et al. (2013). There was no relation, however, between the presence of ulceration and melanocytomas. CAMARGO et al. (2008) showed that 80.95% of melanocytomas did not present ulceration. The presence of ulceration in the epidermis has been associated with short survival. Ulceration is frequently observed in cutaneous melanomas and can be considered an independent prognostic factor (LAPRIE et al., 2001). However, other studies on cutaneous melanomas found in nail beds and lips did not show the same results. In these cases, ulceration did not correlate to the clinical behavior of the neoplasm (SCHULTHEISS, 2006; LACROUX et al., 2012). Also, another study evaluating ulceration in canine oral melanomas was unable to show its prognostic value and survival relevance (HAHN et al., 1994).

We also observed the association between the presence of necrosis and melanotic melanomas. The research by SPANGLER and KASS (2006) showed that the presence of necrosis in melanocytic tumors was associated with shorter survival; however, SMITH et al. (2002) and MILLANTA et al. (2002) considered that the presence of necrosis was unimportant and had no prognostic value. SMEDLEY et al. (2011) stated that the presence of necrosis could represent different biological processes, such as superficial necrosis due to trauma, or deep necrosis due to ischemia.

This study showed that melanomas were associated with nuclear atypia. Aside from tumor location, nuclear atypia is considered one of the main malignancy criteria (MILLANTA et al., 2002; SPANGLER and KASS, 2006), and can provide an accurate prognosis of neoplasm behavior (SPANGLER and KASS, 2006). However, there may be variation among observers and it can be considered a non-quantitative measurement.

Because of this, criteria should be established to maintain reproducibility and promote interobserver concordance (SPANGLER and KASS, 2006; SMEDLEY et al., 2011).

The mitotic index (MI) is the most reliable histological feature to differentiate melanomas from melanocytomas (LIU et al., 2011; CAMPAGNE et al., 2013), showing survival significance independently of other histological parameters as well as location of melanocytic tumors (BOSTOCK, 1979; LAPRIE et al., 2001; SMEDLEY et al., 2011). Our study demonstrated that MI >3/10 HMF was related to malignant melanocytic tumors, corroborating with what was found in the literature, which stated that MI ≥3 mitotic figures per 10 HMF showed malignancy (SMITH et al., 2002; GOLDSCHMIDT and GOLDSCHMIDT, 2017). In contrast, benign melanocytic tumors had MI <3/10 HMF, corroborating GOLDSCHMIDT and GOLDSCHMIDT (2017), who stated that the vast majority of neoplasms with less than 3 mitotic figures per 10 HMF should be considered benign.

The use of immunohistochemical techniques has been widely used in studies of canine melanocytic tumors, because morphological diagnosis alone is insufficient (ROELS et al., 1999; RAMOS-VARA et al., 2000; LAPRIE et al., 2001; MILLANTA et al., 2002; SPANGLER and KASS, 2006; CAMARGO et al., 2008; SMEDLEY et al., 2011; LACROUX et al., 2012; ROLIM et al., 2012). This study used the diagnostic markers Melan-A, HMB-45, and S-100. The Melan-A, also known as MART-1, is one of the most used diagnostic markers in human and veterinary pathology. It is a protein recognized by cytotoxic T lymphocytes (OHSIE et al., 2008; GIUDICE et al., 2010). Some studies related the intensity of Melan-A marking with the biological behavior of the tumor (GIUDICE et al., 2010), because benign behavior neoplasms tend to express moderate to strong marking (CHOI et al., 2003). In contrast, amelanotic melanomas can present poor or even negative marking, as they are related to low cell differentiation and high malignancy (KOENIG et al., 2001; ROLIM et al., 2012). In our study, 95.2% of the cases (20/21) were positive for Melan-A. RAMOS-VARA et al. (2000) reported similar results in oral and metastatic melanomas, because 91.5% of the cases (118/129) were positive, similar to what was reported in humans (BUSAM and JUNGBLUTH, 1999; CHEN et al., 1996; de VRIES et al., 1997).

The S-100 protein is more sensitive than Melan-A, especially for amelanotic melanomas. However, it does not add information about tumor behavior, which justifies the use of an immunohistochemical panel (KOENIG et al., 2001). In addition, it is considered to be less specific because it marks a variety of non-melanocytic cells

(RAMOS-VARA et al., 2000). In our study, we obtained 85.7% positive markings with S-100, similar to other studies that obtained a positivity of 70–76% using the S-100 marker (RAMOS-VARA et al., 2000; Rolim et al., 2012).

The HMB-45 antibody was one of the first melanoma specific markers (BARRA, 2006; OHSIE et al., 2008), with 100% specificity, but with low sensitivity (SMEDLEY et al., 2011). However, SULAIMON et al. (2002) observed that this marker is as sensitive as it is specific for melanomas, rarely presenting cross-reactivity with plasmocytomas. In this study, 81% of the melanomas (17/21) were HMB-45 positive, a number similar with what was found by SULAIMON et al. (2002), who reported 88% of cases that were positive. Distinguishing melanomas from other poorly differentiated tumors is a challenge for pathologists (SULAIMON et al., 2002), thus, it is justifiable to use an immunohistochemical panel (KOENIG et al., 2001). The obtained results in the study corroborated with studies already carried out, and showed that all the markers used had good specificity and sensitivity.

The tumor proliferation index, evaluated through Ki-67 expression, is considered a prognostic factor for human and canine melanomas (LAPRIE et al., 2001). In canine cutaneous melanomas, the proliferative index (PI) is considered low when less than 15% (percentage of positive cells in 500 neoplastic cells), and high when it is 15% or greater (LAPRIE et al., 2001). In canine oral melanomas, PI>19.5 has sensitivity of 87.1%, and specificity of 85.4% in predicting euthanasia or death by oral melanoma within one year after diagnosis (BERGIN et al., 2011). In this study, all melanocytomas had PI <25%, confirming a better prognosis. In contrast, all amelanotic melanomas had higher percentages of PI (>50%). Melanotic melanomas showed PI ranging from negative to greater than 75% positive cells. These data show that amelanotic melanomas have high PI and, consequently, would have a worse prognosis and lower survival.

In humans, TIL can be considered an independent prognostic factor in primary cutaneous melanomas (CLEMENTE et al., 1996; AZIMI et al., 2012; THOMAS et al., 2013). In this type of melanoma, TIL can be characterized as absent, inactive, or active. To be considered active, lymphocytes must be diffusely distributed in the tumor. The inactive pattern refers to TIL with rare lymphocytes, with isolated distribution, or in the periphery of the tumor (CLARK et al., 1989; CLEMENTE et al., 1996). Intratumoral TIL can launch an immune response against the primary melanoma, and is associated with good prognosis (BURTON et al., 2011). This has also been demonstrated in studies that related the abundant presence of TIL with higher survival in humans (CLEMENTE et al.,

1996; BERNSEN et al., 2004; PIRAS et al., 2005; AZIMI et al., 2012; THOMAS et al., 2013).

The regression phenomenon is also related to the presence of TIL, in which the host's immune response can act and result to partial or complete regression of the disease (TAYLOR et al., 2007; AZIMI et al., 2012). In addition, low TIL in the primary tumor indicates metastasis in sentinel lymph nodes in patients with cutaneous melanomas (TAYLOR et al., 2007). Another study, in contrast, by BURTON et al. (2011) states that the relevance of TIL as an independent prognostic factor in cutaneous melanomas remains controversial. In dogs, neither spontaneous remission nor the impact of TIL on prognosis have been reported, and hence, the role of TIL is still hypothetical for this species (ATHERTON et al., 2016).

The presence of TIL, as well as its prognostic significance in dogs, depends on the studied tumor. TIL is associated with tumoral regression in cutaneous histiocytomas (COCKERELL and SLAUSON, 1979; PIRES et al., 2013), whereas in canine mammary tumors, the presence of CD3+ TIL is related to tumor progression and short survival (KIM et al., 2010; CARVALHO et al., 2011). As far as we know, studies on TIL in canine melanocytic tumors are scarce. The study by MONTEIRO (2013, unpublished data) evaluated TIL by CD3 T lymphocytes in canine melanocytic tumors and concluded that malignant melanocytic tumors presented moderate or abundant TIL. The study observed that a higher TIL degree in oral melanocytic tumors was accompanied by inflammation, necrosis, and high MI. It suggested that increased TIL could be associated with increased tumor aggressiveness. In contrast, the study by MARTÍNEZ et al. (2011) found no association between TIL from CD3 lymphocytes and COX-2 expression in canine melanocytic tumors, the latter being strongly related to prognosis and survival.

In our study, we also related the presence of TIL with higher tumor aggressiveness, because we noted an absence of TIL in melanocytomas, which corroborates with MONTEIRO's study (2013, unpublished data), as he found scarce or absent TIL level in melanocytomas, and a moderate to severe TIL level in melanomas. In addition, both of our studies showed different TIL levels in melanotic melanomas, and the highest level (3+) in amelanotic melanomas. This study showed no association between the type of melanocytic tumor and TIL location. The predominant TIL pattern was intratumoral (46.7%), similar to the study by MONTEIRO (2013, unpublished data), which showed the intratumoral pattern in 51.3% of cases. On the other hand,

CLEMENTE et al. (1996) showed a higher frequency of peritumoral TIL in his study on primary cutaneous melanoma in humans.

In this study, immunostaining performed for CD3 and CD20 to identify the TILs present in canine melanocytic tumors revealed numerous CD3+ T cells and the absence of CD20+ B cells in most of the tumors. The role of B lymphocytes in antitumor immunity is well known in other tumors, such as human mammary carcinomas, and the results found in our study are very similar to those reported by MARSIGLIANTE et al. (1999), who observed few B cells comprising TILs (24.4%). B lymphocytes have important functions in the microenvironment of tumors, which are positive when it comes to the control of tumor cells such as the production of antibodies, cytokines, and presentation of antigens, or even via direct cytotoxic action through FasL expression. However, B cells may also act negatively on tumor control, either as regulatory B cells (Bregs), promoting immunosuppressive action, or by producing IL-10, IL-35 and TGF-β, which lead to decreased T cell function (SHEN et al., 2016). This dubious role of B-lymphocytes may be attributed to their state of activation, i.e., resting B lymphocytes promote T cell inhibition, while active B cells stimulate T cell action (RODRÍGUEZ-PINTO, 2005; WATT et al., 2007).

Innate and adaptive immune systems play an important role in tumor immune surveillance, particularly the cell-mediated immunity controlled by T lymphocytes (DIEFENBACH and RAULET, 2002). T cell subtypes can perform different functions in the microenvironment of tumors. CD8+ T cells (cytotoxic) and CD57+ natural killer (NK) cells are able to destroy neoplastic cells (DIEFENBACH and RAULET, 2002; YU and FU, 2006). Furthermore, the T helper cells (Th) (CD4+) and subgroups, which are defined by the type of cytokine they secrete (Th1 and Th2), also participate in the antitumor response, and Th2 lymphocytes are more effective in this response (Yu and Fu, 2006). In contrast, CD4+CD25+ regulatory T cells (FOXP3+) are related to the suppression of effector T cells, and hence, to lower tumor response (SOMASUNDARAM et al., 2002; CURIEL et al., 2004). High Tregs populations have been observed in canine oral melanomas (HORIUCHI et al., 2010; TOMINAGA et al., 2010), which may be related to tumor progression and immune tolerance to tumor cells, suppressing cell immunity (HORIUCHI et al., 2010). In this context, we believe that the CD3+ lymphocytes found in the melanomas of this study may consist of Tregs, which play a regulatory role, inhibiting the antitumor effect. As suggested in other studies (SINHA et al., 2007; HORIUCHI et al., 2010; TOMINAGA et al., 2010), we also believe that effective

immunotherapy for cancer should be based on overcoming or eliminating tolerance to tumors and to immune system evasion mechanisms.

The presence of TIL had a large relationship with ulceration, necrosis, and atypia, which can be used as prognostic parameters in dogs. However, varies among the consulted studies (LAPRIE et al., 2001; MILLANTA et al., 2002; SMITH et al., 2002; SPANGLER and KASS 2006; LACROUX et al., 2012). In several types of cancer, LTI can influence the prognosis, however, further studies are needed to prove the magnitude of its contribution, given that the tumor microenvironment is quite complex and formed by interactions between cells and molecular components (GOODEN et al., 2011; AZIMI et al., 2012; MADEJ et al. 2017).

Based on our results, we can conclude that TIL must be further investigated in order to determine its inclusion as a prognostic factor for canine melanomas. However, it is undeniable that it can be valuable, because it has a strong relationship with malignant tumors and other pre-established prognostic factors, such as necrosis, ulceration, and nuclear atypia. T cells play an important role in the antitumor immune response, and we see research into their subtypes to better understand neoplasm behavior as a future perspective for this study.

FINAL COMMENTS

T cells play an important role in the antitumor immune response. Thus, TIL needs to be further investigated to verify its inclusion as a prognostic factor for canine melanomas.

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