



CD4⁺, CD8⁺, FoxP3⁺ and HSP60⁺ Expressions in Cellular Infiltrate of Canine Mammary Carcinoma in Mixed Tumor

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ABSTRACT

Background: Cancer is a complex process that receive many influences of the tumor microenvironment. The participation of immune system cells and proteins in tumor microenvironment is not yet completely understood. Thus, the aim of this study was to evaluate the infiltrate cellular, subpopulations of T-lymphocytes and HSP60 of canine mammary carcinoma in mixed tumor (CMCMT).

Materials, Methods & Results: Female dogs (n = 20) were selected after Canine mammary tumor (CMT) diagnosis and data were achieved throughout clinical-pathological information. Clinical staging was evaluated and tumor biopsies were processed by histology and cellular infiltrate was performed according criteria and grade. Survival curve were generated by Kaplan-Meier and the lymphocytic infiltrate were compared by Log-Rank followed Chi-Square χ^2 . For immunolabeling it was used anti-CD4, anti-CD8, anti-FoxP3 and HSP60 monoclonal antibodies and were attributed scores from 0 to 3. Clinical-pathological relationship was analyzed using Spearman correlation. This study was approved by the Committee for Ethics in Research using Animals (CEUA-UECE), protocol 12247080-2. Our data showed a mean age of 9.3 years-old, the size of tumors presented more than 5 cm (50%), which were located in inguinal mammary glands (70%), and CMTs shows I (70%) and II (30%) grade. The cellular infiltrate was distributed both in peri and intratumoral regions, dispersed multifocally with moderate intensity and lymphocytes were the major populations found into tumors (n = 826 ± 220). In relationship to cellular infiltrate with CMT grade it was observed that lymphocytes ($\rho = 0.28$) and plasma cells ($\rho = 0.22$) showed a slight positive correlation, and an opposed negative correlation of neutrophils ($\rho = -0.1$) and macrophages ($\rho = -0.38$). CMT presents moderate lymphocytic infiltrate (< 800 lymphocytes), shows higher ($P = 0.01$) survival rates as compared to intense lymphocytic infiltrate (≥ 800 lymphocytes). FoxP3⁺ showed lower intensity while CD4⁺ and CD8⁺ expression were concentrated surrounding of lymphocytic infiltrate tumor region. HSP60⁺ was observed in the inflammatory and tumor cells.

Discussion: Our data are according to a greater risk to the development of breast tumor in old bitches, not castrated and before or after puberty, as well as the use of contraceptives based on progesterone and estrogen. In relation to size of tumor, these findings reinforce that there is a relationship of tumor size with a higher malignancy grade and with a worse prognosis. The predominant tumor location was in the inguinal breasts that is attributed to the high activity of the mammary glands to hormonal stimuli. CMT with low clinical staging are associated with greater overall survival of affected bitches. In relation to tumor microenvironment, it has been reported that heterogeneous populations of the immune system cells often infiltrate the mammary tumors, whose lymphocytes are the main cells. It is suggested that tumor lymphocytosis may be necessary for malignant behavior of the tumor microenvironment. On the other hand, macrophages and neutrophils play an important role that may favor or inhibit the tumor cells development in the tumor microenvironment. In our work, CD4, CD8 and FoxP3 labeling were distributed in peri and intratumoral regions, and consequently, these markers can be used as prognostic for CMT, as well as being a potential target for anticancer therapies. This is the first work that presents results about the participation of HSP60 in CMT, however this data needs further investigation. HSP60 participates as a potent activator of the immune system through its peptides and other HSP types were studied in mammary carcinomas in bitches and presenting results that indicate the association of these proteins with the carcinogenesis process.

Keywords: canine mammary glands, carcinoma in mixed tumor, T-lymphocytes, heat shock protein, immunolabeling.

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INTRODUCTION

The etiology of canine mammary mixed tumor (CMMT) involves multifactors but is still not well understood [17]. Cancer is a complex process controlled by the activation of oncogenes, silencing of tumor suppressor genes and the lack of control of epigenetic events that occur within the cell. In addition, the influences of the tumor microenvironment may participate in both, the progression process and tumor regression [13].

Inflammation is an important process of damaged tissues. The chronic inflammation may facilitate the tumor progression from cytokines and chemokines released by the microenvironment cells, including leukocytes [8]. The role of leukocytes in the tumor microenvironment is not yet fully understood, and several efforts have been made to understand the role of these cells in tumor development. Studies have shown that the lymphocytes presence in mammary mixed tumor (MMT) may be involved in a better or worse prognosis for the patient [1,10].

Under stress conditions, the organism undergoes alterations in the cellular metabolism, leading to the formation of molecules that assist in both the repair and the new proteins production. The heat shock proteins (HSP) are a large family of molecular chaperon proteins [5]. Increased HSP in damaged cells may also aid in cell integrity, since it inhibits the apoptosis [6], and overexpression of the different proteins of this group is related to a poorer prognosis of the patient, including MMT [22].

Thus, the objective of this study, was to evaluate the infiltrate cellular, subpopulations of T-lymphocytes (CD4+, CD8+ and FoxP3+) and HSP60 of canine mammary carcinoma in mixed tumor (CMCMT).

MATERIALS AND METHODS

Animals

For the accomplishment of this work, twenty bitches with varied weight and age, affected by CMCMT were used. The animals were attended at the Unidade Hospitalar Veterinária (UHV) of the Universidade Estadual do Ceará (UECE). All the owners were informed about the study procedures, signing a Free and Informed Consent Form. This study was approved by the Committee for Ethics in Research using Animals (CEUA-UECE), protocol 12247080-2.

The animals were diagnosed by clinical and radiological evaluation and confirmed by cytological and histopathological analysis, according criteria [4] and

tumor grade [9]. Initially, the tumors were submitted to macroscopic analysis, then tumor fragments were collected and submitted to classical histology (H & E) and immunohistochemical analysis. Furthermore, all animals clinical data were also collected for clinical staging based on the described system [20] and follow up was done during one year (365 days).

Cellular infiltrate and immunohistochemical analysis

Cellular infiltrate analysis was performed for its location, in peri and/or intratumoral; its distribution as, focal, multifocal or diffuse; its intensity, as absent (0), mild (1), moderate (2) or intense (3), and quantified in eight random fields, avoiding areas near necrosis (Eclipse E200, 400x magnification)¹ [10]. Thus, two intervals of the lymphocytic infiltrate in CMT were used for data analysis, considering moderate (< 800 lymphocytes) and intense quantity (≥ 800 lymphocytes).

Immunohistochemical analysis were conducted in tumor sections for CD4+, CD8+, FoxP3+ and HSP60+. For this, 5 µm sections were mounted on silanized glass slides and subjected to antigen retrieval process (EnVision TMFlex Target Retrieval Solution High pH Code DM828)² or low pH (Code DM829)² for 20 min at 97°C using the Dako pre-treatment (PT) link module². The endogenous peroxidase activity was inhibited by peroxidase block² for 5 min, and slides received the anti-human CD4, anti-human CD8, anti-human FoxP3 and anti-human HSP60 murine monoclonal antibody³ diluted 1:100 and incubated for 1h, at room temperature. Then, slides were washed three times in phosphate buffered saline (PBS, pH 7.2), and then incubated with the reagent polymer (EnVision TMp Dual LinkSystem/HRP)² for 30 min at room temperature and finally diaminobenzidine (DAB)² for 10 min. The sections were counterstained with Mayer's hematoxylin² and observed by optical microscopy attributing the scores absent (0), mild (1), moderate (2) or intense (3). In order to obtain the scores, all slides were analyzed by two observer and compared to control group.

Statistical analysis

Data were previously subjected to Grubbs test for outliers exclusion. Then, the Kolmogorov-Smirnov test and ANOVA for homoscedasticity and homogeneity evaluation were used. The changes observed in the macroscopic and microscopic analyzes and

the location, distribution and intensity of the cellular infiltrate were expressed as a percentage. Cellular infiltrate analysis was expressed as mean \pm standard deviation. The correlation between the inflammatory infiltrate and the tumor grade was analyzed using Spearman correlation test. In addition, survival curve were generated by Kaplan-Meier estimation method and the two intervals of the lymphocytic infiltrate were compared by Log-Rank followed Chi-Square χ^2 . Statistical significance was considered at $P < 0.05$ and analyses were performed using the software SPSS4. The expressions of CD4+, CD8+, FoxP3+ and HSP60+ were performed semi-quantitatively, being classified into scores (0 to 3).

RESULTS

In this study it was observed that 60% of bitches with CMT were Poodle breed, and the others were non-defined breed, which a higher prevalence of malignancies than benign ones was observed. The mean of age was 9.3 years old, whose bitches were not castrated, four of them had at least one gestation and two received an injectable contraceptive. Macroscopically, it was observed that 50% of the tumors had a size bigger than 5 cm, 30% presented size between 3-5 cm and the remainder with nodules smaller than 3 cm. The predominant tumor location was in the inguinal breasts (70%) and the rest in the abdominal breasts. In seven animals, there was a greater occurrence of the tumor mass in the right antimer. Ulceration was evident in 20% of the tumors and in 60% of cases they had only one tumor formation in the breast, while 40% had two to five mammary chain formations.

All samples evaluated presented histological characteristics of CMT. Foci or nodules of epithelial cells with high pleomorphism and atypical mitosis were seen in the middle of a benign mixed tumor. It was observed that the majority of tumors had a tubular formation index ranging from 10% to 75%, moderate nuclear pleomorphism and mitotic index of 9 to 16 mitosis in 10 high-power field (HPF). The CMT had a grade I histological grade in 70% of the cases and 30% were grade II. It was also observed that only 30% of the cases had areas of tumor necrosis. In the present study, regional metastasis was identified in one animal, and in none of the cases was identified the presence of distant metastases.

Data of the inflammatory infiltrate were presented in table 1. It was observed that most of the cells were distributed both in the peri and intratumoral regions (50%, 70% and 46% for lymphocytes, macrophages, neutrophils, respectively), dispersed multifocally (58%, 63% and 50% for lymphocytes, macrophages and plasma cells) with moderate intensity (70% and 52% for lymphocytes and macrophages) [Table 1]. Lymphocytes were the major populations found in tumors ($n = 826 \pm 220$), followed by macrophages, neutrophils and plasma cells (Figures 1, 2A & 2B).

Evaluating the correlation between the type of inflammatory infiltrate with the CMT grade, it was observed that both lymphocytes ($\rho = 0.28$) and plasma cells ($\rho = 0.22$) had a mild positive correlation. The lymphocyte infiltration was higher ($\rho = 0.045$) in tumors with a high histological grade when compared to low grade. The correlations between the neutrophils and macrophages population with the CMT grade were negative ($\rho = -0.1$ and $\rho = -0.38$, respectively).

When analyze the animals overall survival in relation to the two intervals of lymphocytic infiltrate, it was observed CMT with moderate infiltrate (< 800 lymphocytes) showed significantly higher ($P = 0.008$) survival as compared to those with intense lymphocytic infiltrate (≥ 800 lymphocytes) (Figure 3). The lymphocytic infiltrate reported in the present study showed animals with intense lymphocytic infiltrate (≥ 800 lymphocytes) in CMT.

The results of immunostaining for CD4 and CD8 in CMT are presented in figure 2. The CD4 marker was distributed in the tumor infiltrate in both peri and intratumoral regions. In addition, it was observed that CD4 was more concentrated in the areas surrounding the malignant tumor region, presenting well-defined cellular marking of moderate to intense staining (Figure 2C). Similar to CD4+ labeling, the CD8+ labeling was distributed in both peri and intratumoral regions, especially around the areas of carcinoma, from moderate to intense labeling, but with a higher number of cells compared to CD4+ labeling (Figure 2D).

When evaluating the infiltrating leukocytes profile in mamary carcinoma in bitches, it was demonstrated that animals with CMT without nodal metastasis had a higher ($P < 0.05$) amount of T lymphocytes compared to CMT with nodal metastasis, and that the predominant population in these cases was CD8+ T lymphocytes [10]. On the other hand, animals with

nodal metastasis presented higher ($P < 0.05$) CD4+ T lymphocytes and higher ($P < 0.05$) CD4+/CD8+ ratio. In the present study, it was observed that FoxP3+ labeling were distributed in the intratumoral region, with a mild staining in the inflammatory infiltrate at lymphocytes (Figure 2E).

HSP60+ immunostaining was observed in the cytoplasm of tumor cells, as well as in the different cell populations present in the inflammatory infiltrate, with moderate to intense intensity, independently of the cellular type observed (Figure 2F). This profile was not observed in control samples, with only mild immunostaining in cells of the mammary alveoli, not being observed in myoepithelial cells or in the cells of intra- and extra-lobular ducts.

DISCUSSION

In recent years, the specialty oncology has been gaining great prominence in veterinary medicine. This was due to the great advances in research and in the diagnosis of tumors subtypes, especially those that affect pets. Among the main ones, we can highlight the mammary tumors, which present a large number of reports in the veterinary clinic, especially in older bitches. One of the main challenges for veterinary oncologists is the identification of the mammary tumor and its aggressiveness grade.

In this study it was observed that 60% of bitches with CMT were Poodle breed, and the others were non-defined breed, of which a higher prevalence of malignancies was observed. Our data corroborate with works previously described [7,15]. Age, animal breed and inflammatory infiltrate in the tumor environment are good markers for assessing tumor malignancy [3,7]. The mean of age was 9.3 years old, whose bitches were not castrated, four of them had at least one gestation and two received an injectable contraceptive. Based on this, it is possible to emphasize that these factors contribute to a greater risk of development of breast tumor in old bitches, not castrated and before or after puberty, as well as the use of contraceptives based on progesterone and estrogen [12,24].

In relation to CMT location, our results are according with others works, which reported a higher frequency of CMT in the abdominal and inguinal regions, in relation to the thoracic region [23]. This is attributed to the high activity of the mammary glands to hormonal stimuli, such as estrogen, in addition to

having a greater amount of parenchyma to be stimulated [18]. Our data reinforce that there is a relationship of tumor size with a higher malignancy grade and with a worse prognosis [24].

Regarding the clinical staging, our data varied from I to IV stage, besides it was identified metastasis. CMT was characterized according to World Health Organization [20]. CMT with low clinical staging are associated with greater overall survival of affected bitches [19]. One characteristic of cancer that provides worse clinical staging is the metastasis formation, besides being linked directly to a poor prognosis. Bitches identified with regional or distances metastasis present lower overall survival [19]. Therefore, clinical staging provides important data to aid in the treatment and prognosis of affected animals, and that many CMT are classified with low staging, indicating a less aggressive behavior of this histological type of carcinoma.

In the histological findings (Figure 1 and Table 1), CMT had predominantly grade I. It has been reported that bitches with CMT presents a histological grade from low to moderate tumor [7,10]. This indicates that much of the malignant mixed tumors will hardly be of high grade. These data corroborate with research that evidences CMCMT as a histological type with a better prognosis for the animals compared to other types of mammary carcinoma [4].

Regarding the inflammatory infiltrate in CMT, it was observed that most of the cells were distributed both in the peri and intratumoral regions and lymphocytes were the major populations found in tumors (Table 1 and Figures 1, 2). Mammary tumors are often infiltrated by heterogeneous populations of the immune system cells, whose lymphocytes are the main cells found in the tumor microenvironment [16]. It has been reported that CMT presents moderate-intensity multifocal inflammatory infiltrate consisting predominantly of lymphocytes, and that there was no significant difference when compared to the peripheral and intratumoral areas in relation to the morphological and morphometric characteristics in the different inflammatory infiltrates [10,21]. The intense infiltration of lymphocytes into CMT was associated with histological alterations of aggressiveness, once that was observed higher lymphocyte infiltration in tumours with a high histological grade than of low histological grade [16]. Therefore, it is suggested that tumor lymphocytosis may be necessary for malignant behavior of the tumor microenvironment. The correlations between

Table 1. Distribution, localization and intensity of cellular infiltrate in tumor environment.

		Cellular Infiltrate (%)			
		Lymphocytes	Macrophages	Neutrophils	Plasma Cells
Localization	Peritumoral	-	10	14	50
	Intratumoral	50	20	40	30
	Peri/Intratumoral	50	70	46	20
Distribution	Focal	22	27	70	50
	Multifocal	58	63	30	50
	Diffuse	20	10	-	-
Intensity	Mild	13	38	56	90
	Moderate	70	52	44	10
	Intense	17	10	-	-

Data were expressed as percentage.

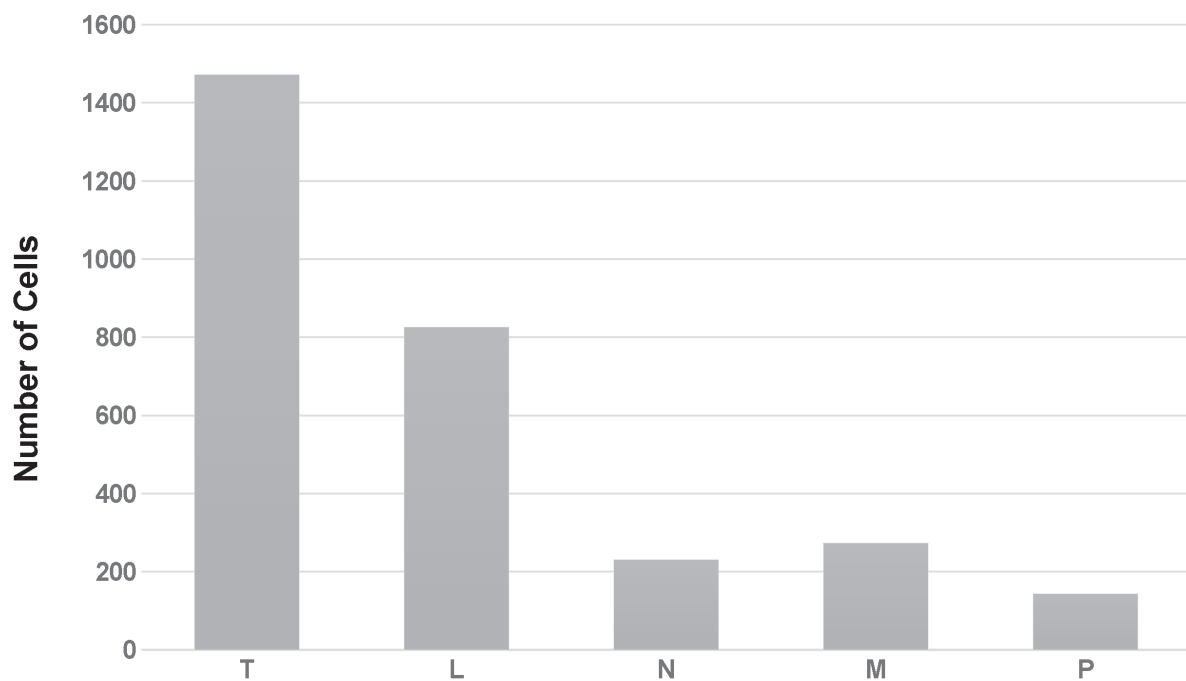


Figure 1. Composition of inflammatory infiltrate in CMT. Total (T), Lymphocytes (L), Neutrophils (N), Macrophages (M) and Plasma Cells (P). Cell count performed on eight fields (400x). Results were expressed in mean.

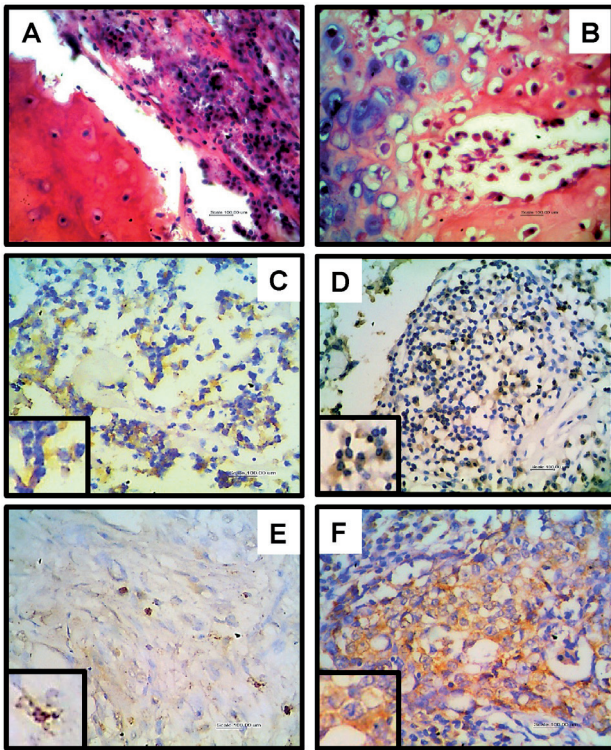


Figure 2. Presence of inflammatory infiltrate in mammary carcinoma in mixed tumor (MCMT) of bitches and CD4+, CD8+, FoxP3+ and HSP60+ expressions in cellular infiltrate. A- Lymphocytes associated with a bone matrix. B- Macrophages between bone marrow and cartilaginous matrix. C- CD4+ T lymphocytes moderately immunostained. D- CD8+ T lymphocytes moderately immunostained. E- FoxP3+ T regulatory lymphocytes mildly immunostained. F- HSP60+ strongly immunostained in tumor and inflammatory cells.

the neutrophils and macrophages populations with the CMT grade were negative. The macrophages and neutrophils in the neoplasias play an important role in the stimulation of the immune system, and depending on the cytokine profile produced, the tumor microenvironment may favor or inhibit the tumor cells development [8].

When analyzing the animals overall survival with two intervals of the lymphocytic infiltrate, it was observed that the CMT have worse overall survival, corroborating with previous studies whose bitches presented mammary tumor. Our data are according to results that the carcinomas with high lymphocytic infiltrate exhibited shorter overall survival [2]. Besides, the high lymphocytic infiltrate can be associated with other poor prognostic factors, such as high histological grade, lymphatic invasion, and necrosis [16].

In the present study, the immunostaining for CD4+ and CD8+ T lymphocytes was evaluated in the inflammatory infiltrate of CMT. Both, CD4+ and CD8+ labeling were distributed around the areas of carcinoma, but predominantly CD8+ labeling. Our data are according with results which demonstrated that CD8+ T lymphocytes predominant population in metastasis cases [10]. Furthermore, the composition of lymphocyte infiltrate in CMT with high proportion of CD4+ T lymphocytes and low CD8+ T lymphocytes

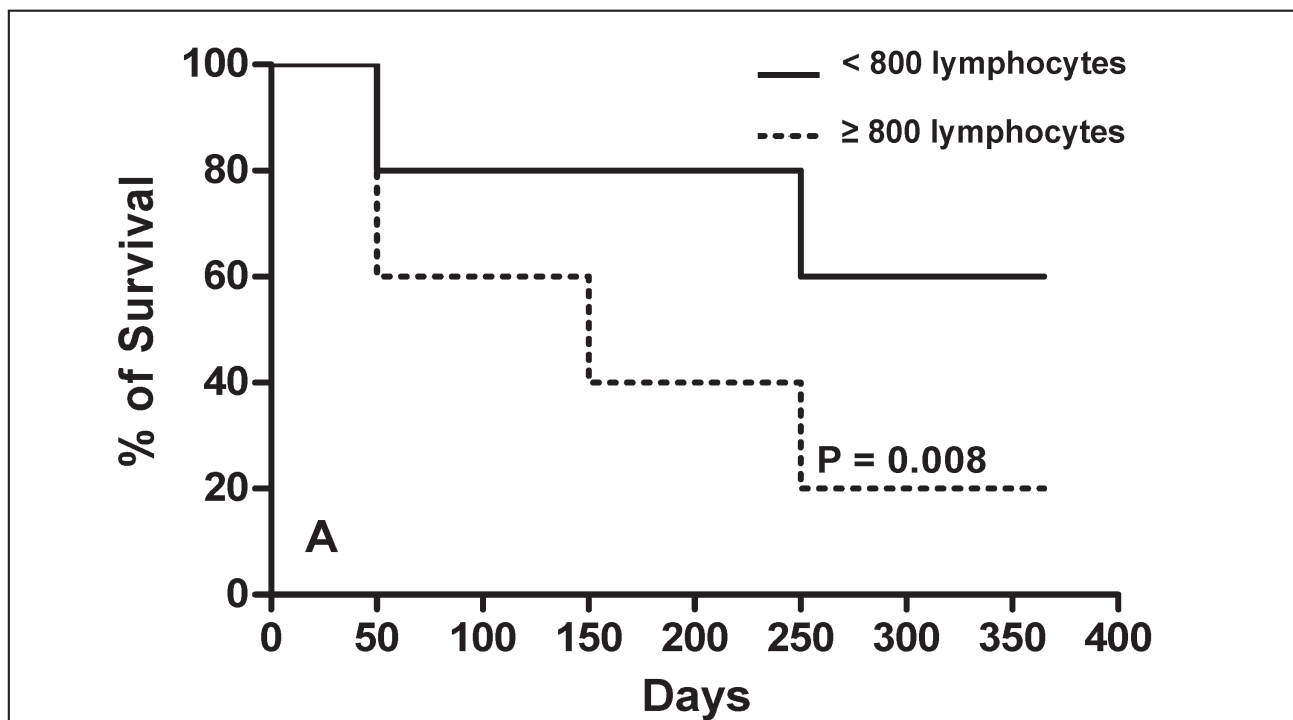


Figure 3. Survival rates of animals with MCMT categorized in two intervals of the lymphocytic infiltrate (< 800 and ≥ 800 lymphocytes).

have a shorter survival time [16]. These results reinforce those found in the present study, suggesting the possibility of using these cells as prognostic biomarkers for CMT, as well as being a potential target for anticancer therapies.

Another evaluated immunostaining was Forkhead box P3+ (FoxP3+). This marker is a transcription factor that is closely linked to regulatory T lymphocytes (Treg) activity and is responsible for characterize this subpopulation of T-lymphocytes in tissues [23]. In the present study, it was observed that FoxP3+ labeling were distributed in the intratumoral region and in the inflammatory infiltrate. The increase of Treg in the tumor microenvironment may be related to factors of poor prognosis of mammary carcinomas, such as high histological grade, lymphatic invasion and necrosis, and lower survival rates for animals [2,16]. In addition, it has been suggested that Treg play a key role in the development of these tumors, since it would be linking immune suppression with tumor angiogenesis, together in the same biological program [1]. Also has been reported the increase of regulatory T cells in the peripheral blood of dogs with metastatic tumors [14].

Heat shock proteins (HSP) belong to a large family of molecular chaperones with the ability to interact reversibly with other proteins, aiding in the formation, folding and trans-membrane transport, besides assisting in the apoptosis process [5]. In our work, HSP60+ immunostaining was observed in the inflammatory infiltrate, in mammary alveoli cells, not being observed in myoepithelial cells or lobular ducts cells. It has been demonstrated that HSP60 participates as a potent activator of the immune system through its peptides [11]. Thus, HSP60 together with their peptides could somehow induce a better response of

lymphocytes to CMT. On the other hand, other HSP types, such as HSP27, HSP72 and HSP90 were studied in mammary carcinomas in bitches and presenting results that indicate the association of these proteins with the carcinogenesis process [22]. However, more studies are needed to understand the involvement of HSP60 in CMT.

CONCLUSIONS

The present study characterized the inflammatory infiltrate in CMT, demonstrating T-lymphocytes as the predominant population. In addition, it was observed that the amount of lymphocytes may be associated with tumor malignancy criteria. CD4+, CD8+ and Foxp3+ markers are present in CMT and their distribution may be associated with the prognosis of bitches. This is the first work that presents results about the HSP60+ participation in CMT, however this data needs further investigation.

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Ethical approval. All procedures and animal care were approved by the Ethical Committee in Animal Use of the State University of Ceará, Brazil (CEUA, UECE) under the Protocol no. 12247080-2.

Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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