Author's response to reviews

Title: Inflammatory response associated with mammary carcinomas in female dogs: immunophenotyping of lymphocytes and the relationships between prognostic factors and survival rates

Authors:

Alessandra Estrela-Lima (aestrela@ufba.br)
Márcio SS Araújo (sobreira@cpqrr.fiocruz.br)
João M Costa-Neto (jmcn@ufba.br)
Andrea Teixeira-Carvalho (andreat@cpqrr.fiocruz.br)
Stela M Barrouin-Melo (barrouin@ufba.br)
Sérgio V Cardoso (cardososv@gmail.com)
Olimdo A Martins-Filho (oamfilho@cpqrr.fiocruz.br)
Rogéria Serakides (serakide@netuno.lcc.ufmg.br)
Geovanni D Cassali (cassalig@icb.ufmg.br)

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To
Rikki Graham, PhD
Senior Scientific Editor
BMC-series Journals
BioMed Central
BMC Cancer

Dear Editor,

We have received your e-mail with the Reviewers’ comments regarding our manuscript, (MS 3699450643033957), entitled “Inflammatory response associated with mammary carcinomas in female dogs: immunophenotyping of lymphocytes and the relationships between prognostic factors and survival rates” by Estrela-Lima and colleagues submitted to the BMC Cancer. We have worked thoroughly to answer all queries raised by the Reviewers, point by point in order to improve the quality of our manuscript and make it suitable to be published at BMC Cancer.

We are presenting below all the queries stated by the Reviewers #1, 2, 3 and 4 as well as the way we have dealt with them to perform the requested changes. The changes were clearly outlined in the revised manuscript, marked in yellow to make it easier for the Reviewers/Editor to access them. We have prepared below a list that responds to the Reviewer’s comments which are highlighted in “bold italic”.
REVIEWER(S)' COMMENTS:

Reviewer #1:

Major:

The major work of this manuscript is the finding of percentages (high or low) of B and T lymphocytes in metastasis and survival rates. It appeared to be preliminary results, and no experiments on the roles of B and T cells in cancer-related inflammation were conducted (but the first two words of the paper’s title “Inflammatory response…” indicates study of relationship between inflammation and tumor). Furthermore, the biological significance of B and T cells in tumor pathogenesis could not be obtained from their data. Current studies have shown that inflammatory cells such as macrophages, mast cells, neutrophils and eosinophils play important roles in cancer-associated inflammation (reviewed in Alberto Mantovani, Paola Allavena, Antonio Sica and Frances Balkwill. Nature 454, 436-444, 2008). However, the authors did not investigate the status of these cells in their tumor samples; this is a big defect in their work.

We acknowledge the Reviewer #1 for this comment. We agree that further investigations are still required in order to clarify the role of distinct leukocyte subsets, including both innate and adaptive immunity cells, in the pathogenesis of mammary carcinomas in female dogs. We are aware that innate immunity cells, such as macrophages, neutrophils and eosinophils may play important cooperative role with adaptive immune response during the establishment of anti-tumor response. Therefore, we are currently processing whole blood samples obtained from female dogs with mammary carcinomas to evaluate the activation status as well as the cytokine profile of innate (neutrophils, monocytes, eosinophils and NK cells) as well as adaptive (T-cell subsets and B-lymphocytes) is order to clarify the role of these cells in the pathogenesis and control mechanisms in canine mammary cancer. We will present these data in another pioneer investigation we intend to submit soon to the BMC-series journal.

We apologize for the misleading title presented in the original version of our manuscript referring as “Inflammatory response associated with mammary carcinomas …”. In order to accomplish this issue, we have changed the title that will appear in the revised version as follows:
“Immunophenotypic features of tumor infiltrating lymphocytes from mammary carcinomas in female dogs associated with prognostic factors and survival rates”.

We are thankful to the Reviewer #1 for the indication of the review from Mantovani et al (2008). We have inserted this outstanding review in the Introduction section of our revised manuscript.

The reference list has been reorganized.

**Minor:**

**Abstract**

1. The conclusion of abstract is obscure and needed to be clarified.
We apologize for the obscure conclusion presented in the original Abstract. We have worked to make it clear in the revised version of our manuscript that appears as follows:

“Conclusion: The intensity of lymphocytic infiltrate and probably the relative abundance of the CD4+ and CD8+ T-lymphocytes may represent important survival prognostic biomarkers for canine mammary carcinomas.”

2. The grammar errors and writing should be corrected and improved in the entire manuscript. For example, “The carcinomas in mixed tumours showed a significantly greater percentage of T lymphocytes than were found in animals without…”

We apologize for the grammar and writing errors. We have worked thoroughly in order to avoid these errors in the revised version of the manuscript. We have counted with a native English speaker to proof read the revised manuscript to minimize the errors. Due to the large number of spelling errors that we have corrected, we have not highlighted them in the revised manuscript.

**Background**
The second paragraph of Background section was directly copied from the first two sentence of Abstract section in the manuscript. They should be modified to make differences.

We apologize for this error. We have decided to change the first sentence on Abstract section that appears in the revised version as follows:

“The immune system plays an important role in the multifactorial biologic system during the development of neoplasias. However, the involvement of the inflammatory response in the promotion/control of malignant cells is still controversial, and the cell subsets and the mechanisms involved poorly investigated.”

Methods
1. Page 6. “Palpitation” should be changed to “palpation”. Latero-lateral right and left (LL) should be replaced by right lateral and left lateral, respectively.

We apologize for these primary errors, probably consequence of mistyping during word processing. We have made the requested changes and also checked the entire manuscript to avoid them throughout the revised document.

2. In the second paragraph of Page 7, the authors stated that tumor tissues were obtained from fragments after bisection of tumor measured as 1.5 X 1.5 cm in size. This indicated intratumoral inflammatory reaction was studied. The authors also described the dynamic interaction between inflammation and tumor development. Accordingly, the dynamic interaction may be different in the areas of intratumor and/or the areas of the periphery. In the reviewer’s opinion, the areas of the periphery should be studied also.

We are thankful to the Reviewer #1 for this comment. We agree that we have investigated the immunophenotypic features of intra-tumor lymphocytes rather than peripheral tumor
inflammatory response. In order to clarify this methodological approach, we have made changes in the revised manuscript, including the Title and Methods section, in order to clarify this issue. We also acknowledge the Reviewer #1 for the suggestion that differences between intra-tumor and peripheral immune response should be addressed. This analysis was performed and appears in the revised version as follows:

“Further comparison between peripheral and intra-tumor areas did not demonstrate any significant differences in the morphological and morphometric features of the inflammatory infiltrates.”

**Result**

*In this study, the involvement of inflammatory cells in the benign tumors was not included.*

We opt did not performed the analysis of inflammatory cells in the benign tumors because in the majority of cases the inflammatory cells found was discrete. Thus, we prioritize the study of inflammatory cells on the carcinomas.

**Discussion**

1. The authors described those parameters including clinical stage, histological type, tumor size, involvement of lymph node metastasis and the mitotic index showed significant correlations with each other. But the reviewer did not find any description of significant correlations between tumor size and lymph node metastasis, or tumor size and mitotic index, or tumor size and histological type.

In order to improve the quality of the manuscript, we opt to reorganize all the Discussion section pointing out a possible association between immunophenotypic features of infiltrating lymphocytes and their relation to prognostic factors and survival.

2. In the third paragraph, types of inflammatory cells secrete which kind of growth factors to promote tumor development needed to be clarified.

See answer for question 1 – Discussion.
Figures

Each data in Figures 3, 4 and 5 should be divided into more panels to make them understandable. And the figure legends should be extended also.

We acknowledge the Reviewer #1 for this suggestion. We agree that the segregation of Figure 3, 4 and 5 into separated panels made them more understandable to readers. Therefore, as requested, we have inserted line borders to define two separated panels on each of these figures and referred to them as top panels and bottom panels in the extended legends.

Other comments:

Level of interest: An article of limited interest.
Quality of written English: Not suitable for publication unless extensively edited.
Statistical review: No, the manuscript does not need to be seen by a statistician.

We have performed all changes suggested by the Reviewer #1 and also counted with a native English speaker to proof read the revised manuscript. We believe that all the changes that we have performed contributed to improve the quality of the manuscript and hope that they have accomplished the above mentioned queries.

Reviewer #2:

This report, discusses the importance of the relative percentage of lymphocyte subpopulations CD4+ and CD8+ in relation to the presence of metastases in canine breast tumours. The paper is worthy of publication, but some minor Essential Revisions are needed; in particular, the authors should better specify the methodologies used in the morphometry of lymphocytes in relation to their identification and quantification.

We acknowledge the Reviewer #2 for these comments and suggestions. As requested, we have better specified the methodologies used in the morphometry of lymphocytes that appear in the revised version of the manuscript as follows:

“The morphometric analysis was carried out in eight selected “Hot Spots” histological fields using an Olympus BX-40 microscope fitted to a 10X eyepiece and a 40X objective, representative
of both peripheral and intra-tumor areas. Morphometric analysis was assessed as the number inflammatory cells per eight fields. Images were captured using an oil immersion 100X objective (a digital camera was adapted to an Olympus BX-40 microscope), and the capture software SPOT version 3.4.5. The inflammatory cells were characterized by image analysis (Corel Draw software version 7.468). Neutrophils, macrophages, lymphocytes and plasma cells were identified based on their morphological features and quantified in HE stained sections. The eosinophils were identified and quantified using additional analysis of Chromotrope 2R staining of serial histological sections [28]. The total number of cells was obtained by adding the eight fields analyzed. The inflammatory infiltrate was first classified as: i) discrete: presence of less than 500 inflammatory cells; iii) moderate: presence of 500-1000 inflammatory cells and iii) intense: presence of more than 1000 inflammatory cells. The cut-off edges were determined based on the mean number of cells obtained during. For data analysis, two intervals were used considering the intensity of the lymphocytic infiltrate: i) discrete + moderate (x< 600 lymphocytes) and ii) intense (≥ 600 lymphocytes), respectively.”

Outros comentários:

Level of interest: An article of importance in its field.
Quality of written English: Acceptable.
Statistical review: No, the manuscript does not need to be seen by a statistician.

We have performed all changes suggested by the Reviewer #2 and also counted with a native English speaker to proof read the revised manuscript. We believe that all the changes that we have performed contributed to improve the quality of the manuscript and hope that they have accomplished the above mentioned queries.
Reviewer #3:
The work signed by Estrela-Lima and others presents, first, results of characterization of inflammatory response associated with mammary carcinomas of the female dog, including immunophenotyping of lymphocytes, and second, results of correlation of inflammatory response and lymphocyte immunophenotyping with prognostic factors and survival. Lymphocytes were the predominant cell type. Differences between lymphocytes subpopulations were found with respect to histological type of tumour, metastasis and survival. The background and design of this study is very interesting and the results force debate concerning the protective-promoting effect of inflammatory cells in neoplasia. Thus, an association between inflammation and indicators of worse prognosis has been found. However, methods are incompletely described and the presentation of the results has some pitfalls which should be addressed in order to clarify their content. In addition, discussion is faint and conclusion vague. To my opinion, the histological classification of tumours, the number of tumours studied by flow cytometry and the statistical study used to analyze the prognostic value of the variables under study are points of major concern. Please find below some hints to improve your manuscript.

MAJOR COMPULSORY REVISIONS

Abstract

1) Methods: Indicate precisely which prognostic factors were analyzed.
The prognostic factors were indicated in the Abstract section that appears in the revised version as follows:

“Fifty-one animals with mammary carcinomas, classified as carcinomas in mixed tumors-MC-BMT=31 and carcinomas-MC=20 were submitted to systematic clinical-pathological analysis (tumor size; presence of lymph node and pulmonary metastasis; clinical stage; histological grade; inflammatory distribution and intensity as well as the lymphocytic infiltrate intensity) and survival rates.”
The prognostic factors were also inserted in the Methods section that appears in the revised version as follows:

“The clinical-pathological parameters, including tumor size, lymph nodes metastasis, pulmonary metastasis, clinical stage, histological grade, inflammation distribution and inflammation intensity, were used during comparative analysis between MC-BMT and MC.”

2) Results:
   a. Indicate precisely the relationship between inflammatory response (intensity, distribution and cellular type) with prognostic factors.
   We apologize for misleading information. In order to solve this query, we have reorganized the data from univariate and multivariate analysis and present them in Tables 1, 2 and 3.

   b. Correct the second sentence where the percentage of T lymphocytes is compared between two different parameters, histological type and metastasis.
   As requested, the text was corrected.

3) Conclusion: Be precise with respect to present results.

We have worked to make conclusion clear in the revised version of our manuscript.

Methods
1) Create a new section listing the prognostic factors studied.
   The list of the prognostic factors was included in the Groups of animals topic - Methods section and appears in the revised manuscript as follows:

   “The clinical-pathological parameters, including tumor size, lymph nodes metastasis, pulmonary metastasis, clinical stage, histological grade, inflammation distribution and inflammation intensity, were used during comparative analysis between MC-BMT and MC.”
2) The mitotic index is not a prognostic factor in canine mammary carcinoma. On the contrary, the number of mitosis is one of the parameters evaluated when establishing the histological grade of malignancy, which is an independent prognostic factor in canine mammary carcinoma (see the chapter written by Lana, Rutteman and Withrow in the 2007 edition of Withrow and MacEwen’s Small Animal Clinical Oncology, Saunders). I suggest calculating the histological grade of the tumours studied.

We thankful the Reviewer #3 for this comment. As requested, we have performed the histological grade. The informations regarding this topic were inserted in the Methods and Result sections.

3) In the morphological and morphometric analysis of the tumour inflammatory infiltrate:

   a. Define how intensity was classified into mild, intense or severe.

   The intensity of the inflammatory reaction was categorized into three subgroups (discrete, moderate or intense) based on morphometric analysis of total inflammatory infiltrate. The inflammatory infiltrate was classified as: i) discrete: presence of less than 500 inflammatory cells; iii) moderate: presence of 500-1000 inflammatory cells and iii) intense: presence of more than 1000 inflammatory cells.

   b. Define the type of staining used and the parameters analysed in the morphometric study of histological fields. As actually presented, it is not clear whether different pictures were taken for the study of eosinophils and the other cell types.

   In order to clarify this issue, we have made alterations in the text that appears in the revised manuscript as follows:
“The morphometric analysis was carried out in eight selected “Hot Spots” histological fields using an Olympus BX-40 microscope fitted to a 10X eyepiece and a 40X objective, representative of both peripheral and intra-tumor areas. Morphometric analysis was assessed as the number inflammatory cells per eight fields. Images were captured using an oil immersion 100X objective (a digital camera was adapted to an Olympus BX-40 microscope), and the capture software SPOT version 3.4.5. The inflammatory cells were characterized by image analysis (Corel Draw software version 7.468). Neutrophils, macrophages, lymphocytes and plasma cells were identified based on their morphological features and quantified in HE stained sections. The eosinophils were identified and quantified using additional analysis of Chromotrope 2R staining of serial histological sections [28]. The total number of cells was obtained by adding the eight fields analyzed. The inflammatory infiltrate was first classified as: i) discrete: presence of less than 500 inflammatory cells; iii) moderate: presence of 500-1000 inflammatory cells and iii) intense: presence of more than 1000 inflammatory cells. The cut-off edges were determined based on the mean number of cells obtained during. For data analysis, two intervals were used considering the intensity of the lymphocytic infiltrate: i) discrete + moderate (x< 600 lymphocytes) and ii) intense (≥ 600 lymphocytes), respectively.”

c. Define the “histological groups” mentioned in line 19 of page 8.
The definition of the histological groups was included in the topic “Groups of animals” – Methods section.

d. Define the “histological groups” mentioned in line 8 of page 10.
The definition of the histological groups was included in the topic “Groups of animals” – Methods section.
4) Survival time:

a. In page 10, lines 15-16, please indicate how were the two intervals defined: by nude eye? by morphometry? Do these intervals correlate with your classification of the inflammatory infiltrate into mild, intense or severe?

The two intervals were defined by morphometry. For data analysis, two intervals were used considering the intensity of the lymphocytic infiltrate: i) discrete + moderate (x< 600 lymphocytes) and ii) intense (≥ 600 lymphocytes), respectively.

5) Statistical methods: Although the correlation between any tumour characteristic and well-known prognostic factors and survival is indicative of the prognostic indicator nature of the characteristic under study, multivariate studies are needed to know the dependent or independent nature of such a variable. Taking into account that the authors have data concerning well-established prognostic indicators (clinical stage, metastasis, tumour size) in addition to survival time, it is not clear to this reviewer why a multivariate statistical study has not been used. I strongly recommend reinforcing the power and quality of the results obtained by performing a multivariate statistical analysis.

We thankful the Reviewer #3 for this suggestion. As requested, we also have performed the multivariate statistical analysis of the data.

Results

1) Taking into account the results presented under “Clinico-pathological evaluation” it appears that the histological classification of tumours you have used is not the WHO classification of Misdorp and others (1999) mentioned in the methods section. Complex carcinoma is “relatively common in the dog” (Misdorp et al 2009). Accordingly, it is hard to believe not a single case was presented in your series.

The histological classification was performed according to the World Health Organization system (WHO) [24] complemented by the proposal of Cassali et al (2002) for classification of
micropapillary carcinoma [25]. Tumors with foci of malignant-appearing cells or distinct nodules of such cell occurring in benign mixed tumors were diagnosed as carcinomas in benign mixed tumors (MC-BMT). Tumors composed of one type of malign cell either resembling luminal epithelial or myoepithelial cells were diagnosed as carcinomas (MC) [24].

As our sample is constituted of tumors larger than five centimeters, presenting carcinomatous areas associated with chondroid and/or osseous matrix, probably these initially benign tumors undergo a malignant transformation of their epithelial component (Misdorp et al., 1999), justifying the diagnosis of carcinoma in benign tumor. No complex carcinoma was observed in our sample. This may also be occurred owing to tumor size, as Erde´lyi et al (Histochem Cell Biol. 2005 Aug;124(2):139-49) showed that the cartilaginous differentiation of complex tumors and myoepitheliomas indicate that the myxoid tissues and myoepithelial-like cell proliferations are the precursor tissues of the ectopic cartilage in mixed tumors.

2) Page 12, lines 7-9: The meaning of this sentence is not clear. I suggest writing it down in another way to make it clear what “histopathological diagnosis” correlates with each parameter.

We apologize for this misleading information. In order to clarify this issue, we have reorganized and rewritten the Clinical-patological analysis and Morphologic and morphometric analysis topics in the Results section.

3) Page 12, lines 9-10: Indicate the parameters with respect to which the differences between the groups (MC-BMT and MC) were found.

We apologize for misleading information. In order to solve this query, we have reorganized the data from univariate and multivariate analysis and present them in Tables 1, 2 and 3.

4) Page 12, lines 15-16: When talking about “distribution of inflammation” clarify which type of those defined in the Methods sections you are referring to (focal, multifocal, or diffuse?)
The distribution of inflammatory infiltrates were evaluated in peripheral and intra-tumor areas and classified as: i) focal: presence of 1-3 inflammatory foci; ii) multifocal: presence of more than 3 inflammatory foci and iii) diffuse: presence of inflammatory cells evenly distributed in the tumor section. The intensity of the inflammatory reaction was categorized into three subgroups (discrete, moderate or intense) based on morphometric analysis of total inflammatory infiltrate.

5) Page 13, lines 10-11: Scores I, II and III have not been defined in the Methods section.

We have included informations about scores that appears in the revised manuscript as follows:

“Histological grade was defined according to Elston and Ellis (1991) [REF] using 4μm HE stained tissue sections. For nuclear pleomorphism, the score #1 was used when the nuclei were small, with little increase in size in comparison with normal breast epithelial cells, and had regular outlines and a uniformity of nuclear chromatin. The score of #2 was assigned when the cells were larger than normal, had open vesicular nuclei with visible nucleoli, and showed moderate variability in size and shape. The score #3 corresponded to marked variation in size and shape, especially when very large and bizarre nuclei were vesicular with prominent and often multiple nucleoli. Strict criteria for identification of mitotic figures were employed according to Diest et al. (1992) [REF]. Mitotic activity was assessed as the number of mitosis cells per 10 fields, performed by two independent analysts in a blinded fashion, using an Olympus BX-40 microscope fitted to a 10X eyepiece and a 40X objective. Using this equipment, one high power field visualizes an area of 0.239 mm2 [26-27]. Tumor up to 7 mitotic figures was scored as 1 point, 8-16 mitotic figures as 2 points and more than 17 mitotic figures as 3 points. Final histological grade was obtained by adding up the scores #1, #2 and #3 and classified as follows: i) grade I: 3-5 points, well differentiated; ii) grade II: 6-7 points, moderately differentiated and grade III: 8-9 points, poorly differentiated.”

6) What was the reason for the lack of flow cytometry analysis in more than half of the tumours (24 analysed, 51 in total)?
The lost of cells during tissue samples processing leading to low cell recovery rates may occur and therefore some samples does not yield enough material for immunophenotyping procedures. In this study, twenty-four out of fifty-one samples were eligible with $2 \times 10^6$ cells and included in the flow cytometric analysis.

7) Page 15, line 10: Enumerate the “classical prognostic factors analysed”.
The classical prognostic factors analysed were listed in the Groups of animals topic in the Methods section that appears in the revised manuscript as follows:

“The clinical-pathological parameters, including tumor size, lymph nodes metastasis, pulmonary metastasis, clinical stage, histological grade, inflammation distribution and inflammation intensity, were used during comparative analysis between MC-BMT and MC.”

Discussion
1) Page 18, second paragraph: To my opinion, there are no data in this work to support this assertion as time between first appearance of symptoms and diagnosis were not recorded. Further, the references are not adequate: This histological type of tumour was first described in literature by Misdorp and others in the last WHO histological classification of tumours of the mammary gland of the dog published up to date (1999).

In order to improve the quality of the manuscript, we opt to reorganize all the Discussion section pointing out a possible association between immunophenotypic features of infiltrating lymphocytes and their relation to prognostic factors and survival.

2) Page 19, second paragraph: Clarify the meaning of the sentence concerning oestrogen synthesis in this context.

See answer for question 1 – Discussion.
3) Page 19, third paragraph: The first two lines should be mentioned in the Methods section also! In addition, when saying “that T lymphocytes represent the predominant fraction in the cellular infiltrate …” the way they were counted should be clarified and also indicated in the Methods section.

See answer for question 1 – Discussion.

4) Page 20, lines 3 and 4: The term “tumour development” when referring to metastasis is confusing. If tumour “development” had been prevented, then the benign mixed tumour would have never become a carcinoma in benign tumour! I suppose you mean “tumour progression”. The same confusing terminology can be found in other parts of the manuscript such as page 20, last paragraph.

See answer for question 1 – Discussion.

5) Page 22, second paragraph: Explain the relationship of this paragraph with your findings.

See answer for question 1 – Discussion.

**Conclusion**

I suggest you to be more precise in your conclusion on the basis of present results. For example, talk about the favourable or unfavourable prognostic value on the inflammatory reaction in mammary carcinoma of the dog instead of mentioning “important factors”.

We are thankful the Reviewer #3 for this suggestion. We have rewritten the Conclusion section that appears in the revised manuscript as follows:

“Our data demonstrated that from the immunophenotypic standing point, the analysis of tumor infiltrating CD4+ and CD8+ T-cells may represent important complementary prognosis biomarkers to be further investigated in canine mammary carcinomas. The higher percentage of infiltrating CD4+ T-cells was observed in animals with the worse prognosis, including those with lymph node metastasis and lower survival in days. On the other hand, the relative percentage of tumor infiltrating CD8+ T-cells were important biomarker that was correlated with higher
survival in days and absence of metastasis in canine mammary carcinomas. The importance of B-cells as a prognostic biomarker still remain to be elucidated, may be approaching further analysis of B-cell subsets, their activation status. Furthermore, additional studies are still needed to expand our understanding of the complex mechanisms involved in the function and interaction of infiltrating CD4+ T-lymphocytes, their cytokine biosynthesis, as they relate to increased malignancy and metastasis, and of the role played by CD8+ T-cells in inhibiting tumor development, determining the tumor microenvironment that favor the control mechanisms in canine mammary carcinomas.”

References

References to basic, fundamental studies on clinical and morphologic prognostic factors of canine mammary carcinoma are lacking. Most of them are already listed in the main books of clinical (Withrow & MacEwen 2007) and pathological (Meuten 2002) Veterinary Oncology.

As requested by the Reviewer #3, references about fundamental studies on clinical and morphologic prognostic factors of canine mammary carcinoma were included in the manuscript.

MINOR ESSENTIAL REVISIONS

1) Page 6, line 9: “palpation” is misspelled.

We apologize for these primary errors, probably consequence of mistyping during word processing. We have made the requested changes and also checked the entire manuscript to avoid them throughout the revised document.

2) Page 7, lines 6-7: Revise the sentence. Do you mean that tumour selected for histomorphometric analysis and immunophenotyping was the larger one in case of multiple tumours?
As suggested by the Reviewer #3, we have rewritten the sentence in the Tumor samples topic – Methods section clarifying that in cases with multiple nodules, the larger tumor size was considered during the classification procedures.

3) The level of significance of statistical tests is presented as “p=” and “p<” with no defined criterion to do so (see for example the third paragraph in page 15 and the last one in page 17). I suggest unifying.

We have revised this issue in all manuscript.

Outros comentários

Level of interest: An article whose findings are important to those with closely related research interests.

Statistical review: No, the manuscript does not need to be seen by a statistician.

We have performed all changes suggested by the Reviewer #3. We believe that all the changes that we have performed contributed to improve the quality of the manuscript and hope that they have accomplished the above mentioned queries.

Reviewer #4:


The paper, by using proper techniques, describes the role of the inflammatory/immune response in the progression of canine mammary tumours, allowing to get prognostic indications from the cell types present and their distributions. The paper is clear in the aims but both the introduction and the discussion are too much devoted to human results and lack
of the information available on this topic in the veterinary field. Some points of the text in the material and methods and results sections needs to be improved as follows.

**Major Compulsory Revisions**

**Abstract**

1. What does “pure carcinoma” means? Simple carcinoma or complex carcinoma?

We apologize for this wrong nomenclature. In fact, it was simple carcinoma. We have changed the wrong nomenclature and used the term “carcinoma” to refer to this type of tumor.

**Background**

2. Rewrite the sentence “It is believed that, in certain situations, the cells responsible for modulation of the inflammatory response release autocrine and paracrine factors that stimulate cellular proliferation and angiogenesis, as well as inhibiting apoptosis, thus altering the immune response to aggression [7;13].” It is not comprehensible.

We are thankful the Reviewer #4 for this comment. As requested, we have rewritten the sentence that appears in the revised manuscript as follows:

“In certain situations, the cells responsible for modulation of the inflammatory response release chemokines and cytokines that stimulate cellular proliferation and angiogenesis, as well as inhibiting apoptosis, thus altering the immune response to aggression [7;13-14]. There is evidence that major inflammatory cytokines (such as IL-1-β, IL-6, IL-23 and TNF-α) promote tumor development by acting directly or indirectly on neoplastic cells [9;14;37]. These factors together can accelerate mutagenesis and promote the survival of atypical clones with a greater capacity to invade tissues and organs [11].”
Methods

3. **Change palpitation with palpation.**

We apologize for these primary errors, probably consequence of mistyping during word processing. We have made the requested changes and also checked the entire manuscript to avoid them throughout the revised document.

4. **Why only chest radiographs were used to follow up the animals?**

All animals included in the survival rate analysis were submitted to quarterly follow-ups during twelve months, including systematic clinical evaluation, radiological examinations along with biochemical and hematological analysis.

5. **“In cases with multiple nodules, the tumour was classified as a larger tumour for the histomorphometric analysis and immunophenotyping.” This way is not correct because in case of multiple nodules you should consider only the larger, otherwise a bias is referred to all the smaller nodules if they are not related to the largest. In the dog often the mammary tumours consist of multiple nodules not related each other.**

We have rewritten the sentence in the Tumor samples topic – Methods section clarifying that in cases with multiple nodules, the larger tumor size was considered during the classification procedures.

6. **How where WHO and Cassali et al classification systems merged or integrated?**

The tumor samples were classified based on the histopathological diagnosis according to Misdorp et al. (1999) complemented by the proposal of Cassali in 2002 (Arq. Bras. Med. Vet. Zootec;54(4):366-369, July-Aug. 2002). These authors described the morphological and immunohistochemical findings of two cases of mammary invasive micropapillary carcinoma occurring in dogs. Histologically, the tumors are characterized by the presence of numerous
irregular cystic formations filled out with nests of epithelial cells that exhibit a micropapillary pattern. These morphological features are characteristic of invasive micropapillary carcinoma in woman, a mammary tumor not previously described in dogs. Gama et al, in 2008, comments about the clinicopathologic features of mammary invasive micropapillary carcinoma (IMC) in dogs (Vet Pathol. 2008 Jul;45(4):600-1). This type of tumor was described in cats in 2007 (Vet Pathol. 2007 Nov;44(6):842-8).

7. It is correct to refer mitotic index to 10 fields, each with the same area, but the data is not corrected for cellularity. Four mitoses in a field of 0.239 mm² of a solid carcinoma are lower than four mitoses in a field of 0.239 mm² of a papillary carcinoma. Correction of the data for cellularity must be considered, otherwise the data are not comparable between different types of tumours.

In breast cancer, the Elston-Ellis Modification of the Scarff-Bloom -Richardson System provides more objective criteria for three component elements of grading and specially addresses mitosis counting in a more rigorous fashion. In this system, the authors do not discriminate the type of tumors. These modifications have enhanced reproducibility of grading among pathologists. Because of variations in field size, the size must be determined for each microscope and the appropriate point score determined accordingly (Please see: Arch Pathol Lab Med. 2000; Arch Pathol Lab Med. 2009).

8. The objective used to capture the images needs to be reported.

We are thankful the Reviewer #4 for this suggestion. As requested, we inserted this information in the Methods section that appears in the revised manuscript as follows:

“Images were captured using an oil immersion 100X objective (a digital camera was adapted to an Olympus BX-40 microscope), and the capture software SPOT version 3.4.5.”
9. Was only for eosinophil counts the chromotope 2R stain used? Were all the other cell types counted on H&E stained sections? Please specify.

The informations requested above were inserted in the Methods section and appear in the revised manuscript as follows:

“Neutrophils, macrophages, lymphocytes and plasma cells were identified based on their morphological features and quantified in HE stained sections. The eosinophils were identified and quantified using additional analysis of Chromotrope 2R staining of serial histological sections.”

10. For the quantitative variables the test used to identify normal or not normal distributions of the data must be reported.

The information requested was inserted in the Statistical topic - Methods section. For the quantitative variables the Kolmogorov-Smirnov test was used in order to identify normal or not normal distributions of the data.

11. The Kaplan Meyer are curves and the COX is a test. Did you use the log rank test or the COX test to compare the curves? 0.05 was the alpha level used for all the test or only for the log rank test? Which was the alpha level for the other comparisons?

The survival curves were estimated with the Kaplan-Meier estimation method followed by Log-rank test. The multivariate analysis was carried out using the proportional hazards test (COX model). The values were considered statistically significant when p <0.05.
Results

12. “The carcinoma group consisted of, in increasing degree of malignancy, three tubular carcinomas, four papillary, three tubular-papillary, four solid (Fig. 1B), three micropapillary, two anaplastic and one special type called mucinous carcinoma.” The sentence does not respect the tumour groups reported in the WHO classification. It is considered a tubular-papillary carcinoma group and not 3 different types (tubular, papillary and tubular-papillary), the increasing malignancy is in the progression tubular-papillary, solid and anaplastic carcinomas. How many were the in situ carcinomas?

The expression “increasing degree of malignancy” was excluded of the sentence. The tumor samples were classified based on the histopathological diagnosis according to Misdorp et al. (1999) [24] complemented by the proposal of Cassali et al (2002) for classification of micropapillary carcinoma [25] and the animals divided into two groups: i) carcinomas in benign mixed tumors (MC-BMT, n=31) and ii) carcinomas (MC, n=20). Histological data reevaluated that MC group comprised distinct subtypes, including tubular (n=3), papillary (n=4), tubular-papillary (n=3), solid (n=4), micropapillary (n=3), anaplastic (n=2) and one special type referred as mucinous carcinoma, characterized by abundant mucin production. In the WHO classification a tubulopapillary carcinoma is characterized by the formation of tubules and/or papillary projections. This category can be subdivided into: (1) tubular carcinoma, those are without papillary elements, and (2) papillary carcinoma, those without tubular components.

In the study samples there no were in situ carcinomas.

13. “The histopathological diagnosis showed a strong inverse correlation with the mitotic index (p = 0.005), survival (p = 0.009), and range of total inflammatory infiltrate (p = 0.025).” The sentence is not correct because the histopathological diagnosis is a qualitative variable. Which is the explanation of an inverse correlation between a qualitative and a quantitative variable?

We apologize for this mistake. These correlations were excluded of the manuscript. We apologize for misleading information. In order to solve this query, we have reorganized the data regarding univariate and multivariate analysis and present them in Tables 1, 2 and 3.
14. “There was significant difference between the groups (MC-BMT and MC) (Fig. 2).” The sentence is not clear. The difference was significant for which values?

In order to improve the quality of the manuscript the Figures 2, 3 and 4 and the text related to them were revised and reorganized.

15. “Tumour size presented a strong positive correlation with stage (p < 0.001), ……….”. The result is expected because the size of the tumour give a great contribution to the final clinical stage in the TNM system, so this analysis should be erased.

These correlations were excluded of the manuscript. In order to solve this query, we have reorganized the data regarding univariate and multivariate analysis and present them in Tables 1, 2 and 3.

16. What are score I and II? They are not presented in materials and methods.

Histological grade was defined according to Elston and Ellis (1991) [REF] using 4μm HE stained tissue sections. For nuclear pleomorphism, the score #1 was used when the nuclei were small, with little increase in size in comparison with normal breast epithelial cells, and had regular outlines and a uniformity of nuclear chromatin. The score of #2 was assigned when the cells were larger than normal, had open vesicular nuclei with visible nucleoli, and showed moderate variability in size and shape. The score #3 corresponded to marked variation in size and shape, especially when very large and bizarre nuclei were vesicular with prominent and often multiple nucleoli. Strict criteria for identification of mitotic figures were employed according to Diest et al. (1992) [REF]. Mitotic activity was assessed as the number of mitosis cells per 10 fields,
performed by two independent analysts in a blinded fashion, using an Olympus BX-40 microscope fitted to a 10X eyepiece and a 40X objective. Using this equipment, one high power field visualizes an area of 0.239 mm² [26-27]. Tumor up to 7 mitotic figures was scored as 1 point, 8-16 mitotic figures as 2 points and more than 17 mitotic figures as 3 points. Final histological grade was obtained by adding up the scores #1, #2 and #3 and classified as follows: i) grade I: 3-5 points, well differentiated; ii) grade II: 6-7 points, moderately differentiated and grade III: 8-9 points, poorly differentiated.

17. “The mitotic index presented a strong correlation with ………., histological diagnosis (p = 0.005) and ……..” How was the correlation calculated and what does the result mean?

These correlations were excluded of the manuscript. We have opted to reorganize the data focusing univariate and multivariate analysis and presenting them in Tables 1, 2 and 3.

18. Change mononuclear lymphocytes in lymphocytes.

We have made the required change in the text.

19. “The proportion of lymphocytes showed a significant and positive correlation with the distribution (p = 0.042) and type of inflammatory response (p = 0.004), and ……….” Explain what does it mean or which distribution and type of inflammatory response was associated with the proportion of lymphocytes.

These correlations were excluded of the manuscript. We have opted to reorganize the data focusing univariate and multivariate analysis and presenting them in Tables 1, 2 and 3.
20. Please refer (or give a table of) the median values for each group before starting the presentation of the results of the survival time paragraph.

We are thankful the Reviewer #4 for this suggestion. As requested, we have included this paragraph the result section.

“The minimum survival was 25 days post surgery and the maximum survival period was 307 days. The median survival is given when 50% of the members of a group die or have a recurrence. Only the metastasis group MC reached the median survival period, showing also lower survival period (183.5 days)”.

21. What are intervals 1 and 2? They are not presented in materials and methods.

For data analysis, two intervals were used considering the intensity of the lymphocytic infiltrate: i) discrete + moderate ($x < 600$ lymphocytes) and ii) intense ($\geq 600$ lymphocytes), respectively.

22. “The survival rate showed a significant and inverse correlation with ……. histological diagnosis ($p = 0.009$), ……………………….” Please explain.

This correlation was excluded of the manuscript. We have opted to reorganize the data focusing univariate and multivariate analysis and presenting them in Tables 1, 2 and 3.

Discussion

23. Explain the difference of results obtained in this study with other in the veterinary field on the same topic.
In order to improve the quality of the manuscript, we opt to reorganize all the Discussion section pointing out a possible association between immunophenotypic features of infiltrating lymphocytes and their relation to prognostic factors and survival.

24. The mechanisms of B, CD4+ and CD8+ cells in the control of tumour should be briefly reported.

As requested, we have made the alterations in the discussion section.

25. “This finding probably results from the extensive areas of ulceration associated with the larger tumours, mainly occurring in this group, which results in bacterial infection and neutrophil recruitment. These cells are also attracted to the tumour site by cancer cells secretion of GM-CSF. The neutrophils also produce oncostatin M (IL-6 cytokine family). When neoplastic cells bind to this cytokine, this induces the secretion of VEGF from the neoplastic cells, which, in turn, increases tumour invasiveness and indicates a poorer prognosis [44].” Erase these sentences because the cell type is not important.

We are thankful the Reviewer #4 for this suggestion. As requested, we have excluded this paragraph of the discussion section.

26. “Macrophages are cells that belong to the mononuclear phagocytic system, and are derived from circulating monocytes [45]. These cells migrate into tissues, where they undergo differentiation into two cell types, M1 and M2, with distinct features. The M1 macrophages are activated by INF-γ and are potent effector cells in fighting tumours. This is very distinct from M2 macrophages, which promote proliferation by producing growth factors related to angiogenesis, tissue repair and remodelling [46].” Erase these sentences because they do not discuss the results presented.
We are thankful the Reviewer #4 for this suggestion. As requested, we have excluded this paragraph of the discussion section.

**Conclusion**

27. **Refer a short conclusion also on the role of the B cells.**

Our findings demonstrated that animals from the MC-BMT group with lymph node metastasis presented higher percentage of tumor infiltrating B-lymphocytes. The role of tumor infiltrating B-cells are still controversial. Some studies have demonstrated that chronic activation of B-cells contributes to tumor development by inhibiting the activity of CD4+ T-cells by yet unknown mechanisms [44]. However, our data demonstrated that together with the enhanced levels of B-cells, there was high frequency of CD4+ T-cells was observed in animals with worse prognosis in MC-BMT tumors. Moreover, other studies have pointed out that, the acute activation of B-cells may have a key role in early elimination of neoplastic cells, through the secretion of antigen-specific immunoglobulins, thus participating in the regression of tumors. Together, these findings demonstrated that the search for B-cell-related biomarkers to canine mammary carcinoma still needs further investigation to provide a more conclusive hypothesis. The paragraph above was inserted in the Discussion section.

Furthermore, we have opted to insert in the Conclusion section a sentence highlighting this aspect that appears in the revised version as follows:

“The importance of B-cells as a prognostic biomarker still remain to be elucidated, may be approaching further analysis of B-cell subsets and their activation status.”

**Minor Essential Revisions**
28. Due to the presence of misspelled words, the text needs the editing of an English mother tongue.

We have performed all changes suggested by the Reviewer #4 and also counted with a native English speaker to proof read the revised manuscript. We believe that all the changes that we have performed contributed to improve the quality of the manuscript and hope that they have accomplished the above mentioned queries.

Other comments:

Level of interest: An article of importance in its field.

Quality of written English: Needs some language corrections before being published.

Statistical review: Yes, and I have assessed the statistics in my report.

We have performed all changes suggested by the Reviewer #4 and also counted with a native English speaker to proof read the revised manuscript. We believe that all the changes that we have performed contributed to improve the quality of the manuscript and hope that they have accomplished the above mentioned queries.

We believed that the requested changes and the contributions of the Reviewers #1, #2, #3 and #4 have improved the quality and understanding of our manuscript. We would like to thank the BMC-series journals members for your attention in revising this article and hope that it is now compatible with the high quality of BMC Cancer and that the revised version is now acceptable for publication in this journal.

Sincerely yours,

Dr. Geovanni Dantas Cassali
Corresponding author