Author's response to reviews

Title: Columnar cell lesions of the canine mammary gland: pathological features and immunophenotypic analysis compared with the human breast

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Author's response to reviews: see over
Dear Dr. Sabina Alam, PhD
Scientific Editor
BMC series journals

Enclosed please find the revised version of our manuscript entitled "Columnar cell lesions of the canine mammary gland: pathological features and immunophenotypic analysis", which has been modified according to the acknowledged reviewer’s suggestions. The following contains our responses to the reviewers’ comments.

After revision the manuscript was edited by a native-English speaking colleague to become adequate for resubmission. Please note that the title was modified. We would like to thank the referees for the attention on revising this article and inform that all changes have been carefully made following the reviewer’s considerations. We believe that all comments and requested changes from the referees have improved the quality of our manuscript. We hope that the revised version is now compatible with the high quality of BMC Cancer, considering the resubmitted version appropriate for publication.

Best regards,
Geovanni Dantas Cassali
Reviewer: Rohit Bhargava
Reviewer's report:
This study describes the presence of columnar cell lesions in canine mammary tissue and its resemblance to human breast tissue. The manuscript is well written; however, several minor essential revisions are required.
1) The authors state that this study was performed on 126 consecutive canine mammary glands. How were these 126 mammary glands obtained? Are these from an autopsy series? Were these removed for mass lesions, etc? Knowing the reasons for mammary gland removal will help estimate the true prevalence of columnar cell lesions in the canines.

The 126 mammary glands samples were obtained from the Veterinary Teaching Hospital of the Federal University of Minas Gerais, after clinical diagnosis of mammary tumor and surgical removal of the lesion. Approximately 1500 mammary tumors were diagnosed during the period of analysis (1998 to 2009). Since this work was based on the review of 126 cases with previous diagnosis of epithelial hyperplasia, it is not possible to estimate the prevalence of columnar lesions. In order to estimate the real prevalence of tumoral lesions, a morphological review of all cases attended in the Veterinary Teaching Hospital in a period of 10 years will be done (approximately 1500 cases), which consists on a future study of our group.
We have completed the information. Please see manuscript.

2) In the results section, it is mentioned that in-situ carcinomas were more frequently observed with FEA. It will be more helpful to know the type of in-situ carcinoma associated with all the columnar cell lesions, i.e. ductal versus lobular, low-grade DCIS versus high-grade DCIS and if there was any statistical difference between types of in-situ carcinoma associated with FEAs. Similarly, it will be useful to know the subtype and receptor status of associated invasive carcinomas. Please also provide the details of type of benign tumors associated with columnar cell lesions. These details with enhance the manuscript and will provide a better understanding of lesions associated with columnar cell lesions.

The DCIS presented in the canine mammary glands are non invasive ductal lesions of high pleomorpfism and low mitotic index. We believe that the identification of FEA associated to DCIS may allow future studies comparing the different types of DCIS in the presence of FEAS, however, this was not our work objective.
We have completed the information about the details of type of benign tumor associated.
Please see manuscript (Table 1).
3) The authors also suggest that dogs are a suitable model for the comparative study of non-invasive breast lesions. However, this is difficult, if not impossible as there are huge challenges associated with use of dogs as laboratory animals. The authors may want to expand on this statement within a paragraph in the discussion section.

*The use of the canine model is based on the study of spontaneous mammary lesions mainly, which is difficult to reproduce in other species. Different studies report the use of female dogs for prognostic and predictive evaluation. There is not an ideal animal model for comparative studies, but we believe that the use of the canine species could be the most adequate model for new therapeutic studies for breast cancer, due to the similarities of human mammary lesions.*

*We have completed the information. Please see manuscript.*

4) Table 1 is confusing. Please break it down to 2-3 tables and provide details of all the associated lesions.

*Ok. Modified. Please see Table 1 and 2.*

5) Typographical errors:
5A) Page 7, last paragraph, 2nd line: Please change “The presence such lesions” to “The presence of such lesions”.

*Ok. Modified.*

5B) Page 8: Correct the spelling for Abbreviations.

*Ok. Modified.*

5C) Legend for figure 4: Please correct typographical errors in the bold text.
5D) Figure 4: Please correct spelling for “without”. Is HCC same for CCH? If yes, then change it to CCH.

*Figures 4 and 5 were eliminated. Please see Table 1 and 2.*

6) Finally, please delete "compared with the human breast" from the title. This gives a wrong impression that human breast tissue was also examined in this study.

*Ok. Modified.*
Reviewer: Adelina Gama
Reviewer's report:

Major Compulsory Revisions
Although the authors have applied the human breast classification, the chosen title: “Columnar cell lesions of the canine mammary gland: pathological features and immunophenotypic analysis compared with the human breast” implies a comparative study using both canine and human mammary samples. It would be more accurate the following title: “Columnar cell lesions of the canine mammary gland: pathological features and immunophenotypical analysis”.

**Ok. The title was modified, another reviewer made a similar suggestion.**

In the 2nd paragraph of the Background section, the authors state: “CCLs to be divided into three broad categories: columnar cell change (CCC), columnar cell hyperplasia (CCH), and further subclassifications of these according to the absence or presence of cytological atypia, collectively known as flat epithelial atypia (FEA).”. This sentence on the classification of columnar cell lesions is not clear and should be clarified. There are two (not three) broad categories: columnar cell change (CCC) and columnar cell hyperplasia (CCH), each further subclassified according to the absence or presence of cytological atypia; the lesions of CCC and CCH with atypia are collectively known as flat epithelial atypia (FEA).

**Ok. Modified.**

Material and Methods: despite 67 identified columnar cell lesions, the authors performed the immunohistochemical analysis in a rather small number of cases. Besides, there is a lack of information on which columnar cell lesions were used. In the 2nd paragraph of Material and Methods section, the authors should clarify the classification applied in the present study. In the background section, CCC referred to Columnar Cell Change, but in this paragraph CCC is used for Columnar cell lesions without atypia? Please clarify.

*The immunohistochemical analyses were procedured only on cases with enough material, considering the small size of the lesions. The objective of the present work was to describe the immunophenotypic characteristics of these lesions. In future works, after reviewing a larger number of cases, we aim to study the immunophenotypic characteristics and correlate them with other associated morphological alterations. The classification applied was modified. Please see manuscript.*

In the 2nd paragraph of Results section, the authors should state “CCL without atypia were identified in 45/67” and not “CCC without atypia…” and then describe both CCC and CCH without atypia.
In the 2nd paragraph of the Results section, the authors state that columnar lesions without atypia were frequently associated with microcalcifications. How many cases were there with microcalcifications? And what about FEA cases? Were there any microcalcifications?

The cases were reviewed and reported in the article. Please see manuscript.

In the 4th paragraph of Results section, the authors state: “Interestingly, in situ carcinoma was more frequently observed in CCLs with atypia (FEA): 11/20 (Fig. 4)”. Although this is correct, this statement is misleading, given that 9 out of 20 in situ carcinomas were also associated with CCLs without atypia. Please comment.

We believe that the percentage of FEAs associated with in situ carcinomas is higher than other lesions. However, the small number of lesions evaluated in the present work is not capable of demonstrating this behavior. A future proposes of analysis with a larger number of FEAs cases may show a more evident relation.

The information regarding statistical associations is rather confusing. Instead of presenting figures (4 and 5), the authors should present their data in a table format, providing the number and percentage of each group of lesions, in addition to the P value.

Ok. Modified. Please see manuscript.

The authors are advised to check the number of cases throughout the Results section, given that they do not always match with the numbers presented in the table. For example, the authors state: “CCCs were detected in association with 23 benign tumors…”, but in Table 1 the authors only mention 21 benign tumors.

Ok. Modified. Please see manuscript.

The last sentence of the Discussion is confusing. Please rephrase it. Ok. Modified, another reviewer made a similar suggestion.
Table 1: the authors should remove the columns on DH. This additional information should be given in the text.

*Ok. Modified. Please see manuscript.*

Figure 3A is out of focus and Figure 3B should be replaced by a more illustrative one. The authors must be aware that this is the first report of columnar lesions in canine mammary gland samples, and thus, it is important to present outstanding microphotographs.

*Figure 3A was modified. We maintained Figure 3B which identifies the columnar epithelial cells lining the acini with intraluminal secretions, showing cytological atypia, high clear/cytoplasmic ratio and microcalcification.*

Please eliminate Fig 4 and Fig. 5 (see comments above). In addition, the authors are advised to use a uniform nomenclature in the text and in table/figure legends. For example, the term “usual ductal hyperplasia” (UDH) is used in Fig. 5 legend but has not been mentioned in the text.

*Figures 4 and 5 were eliminated. Please see Table 1 and 2.*

Minor Essential Revisions

In the abstract, the following sentence “characterized at dilated acini lined with a single layer of columnar epithelial cells” should be corrected, since the authors describe some columnar lesions with more than one epithelial cell layer (as described in the 2nd paragraph of the introduction and in the 2nd paragraph of the results section).

*Ok. Modified. Please see manuscript.*

In the abstract, the sentence “The proliferation rate as measured by Ki67 appeared higher in the lesions analyzed.” is not very clear.

*Ok. Modified. Please see manuscript.*

In Material and Methods, Ki-67 immunoreactivity scoring should be clarified.

*Ok. Modified. Please see manuscript.*

In the Results section, the sentence “Associated lesions were represented only by ductal and lobular hyperplasias.” is not correct, since the authors also describe associated neoplastic lesions (Table 1).

*Ok. Modified. Please see manuscript.*
In the 2nd paragraph of the Discussion, please introduce the missing reference on “Hyperplastic breast lesions, such as duct hyperplasias without and with atypia, have previously been described in the canine mammary gland.”

*Ok. Modified. Please see manuscript.*

In the sentence: “Additional studies are needed to analyze the frequency of columnar cells” (last paragraph), did the authors mean columnar cell lesions?

*Ok. Modified. Please see manuscript.*

Discretionary Revisions
The authors should check the English language throughout the paper, in order to improve the readability of the manuscript.

*The manuscript was edited by a native-English speaking colleague.*
Reviewer: Eriko Tokunaga
Reviewer's report:
In this study, the authors investigated the columnar cell lesions (CCLs) of the canine mammary gland. The finding that CCLs of the canine mammary glands closely resemble CCLs in human specimens is interesting. They concluded that dogs are suitable model for the comparative study of noninvasive breast lesions; however, the scientific impact of this study is poor.

#1. The association between CCLs and coexistence of neoplastic lesions is not clear. Table 1 is difficult to understand. 
Ok. Modified. Please see Table 1 and 2.

#2. The importance of the immunohistochemical analyses, ER, PgR, 34#E-12, E-cadherin, Ki-67, HER2 and p53, is not clear.

The markers used in the present work are well established in the human literature for characterizing the columnar cell lesions, besides its recognition for prognostic and predictive molecular markers of neoplastic and non neoplastic mammary lesions.


#3. The author should describe the criteria of the positivity of Ki-67. 
Ok. Modified. Please see manuscript.

#4. The author should describe what kinds of benign lesions are included. 
Ok. Modified. Please see manuscript.

#5. The entire paper needs to be edited by a native English speaker.

The manuscript was edited by a native-English speaking colleague.
Reviewer: Gulisa Turashvili
Reviewer's report:
- Major Compulsory Revisions

Abstract:
1. Columnar cell lesions (CCLs) are believed to be a non-obligate, intermediate step in development of some forms of low grade DCIS and invasive carcinoma (AJSP 2005;29:734), ie CCLs may be involved in the development of breast cancer rather than in the progression of already developed breast cancer. The same applies to Para 1 in Background.

Ok. Modified. Please see manuscript.

2. ‘The proliferation rate as measured by Ki67 appeared higher in the lesions analyzed’. Please clarify the sentence. Are you comparing normal TDLUs with CCLs?
Ok. Modified. Please see manuscript.

Background:
1. CCLs may be lined (not outlined) by one, two or more (not just one or two) layers of columnar epithelial cells. The number of cellular layers determines whether it is columnar cell change (1-2 cell layers) or columnar cell hyperplasia (>2 cell layers). Also, only non-atypical CCLs show nuclei oriented perpendicular to the basement membrane. The term FEA (flat epithelial atypia) is only used when cytological atypia is present. Please reword Para 2.

Ok. Modified. Please see manuscript.

2. CCLs may be precursors not only for low-grade DCIS but also invasive carcinoma.
Ok. Modified. Please see manuscript.

3. It is not clear how ‘a clearer account of the alterations in canine mammary cancer will almost certainly lead to a better understanding of the key steps in the formation of human tumors’.

The use of the canine model is based on the study of spontaneous mammary lesions mainly, which is difficult to reproduce in other species. Different studies report the use of female dogs for prognostic and predictive evaluation. There is not an ideal animal model for comparative studies, but we believe that the use of the canine species could be the most adequate model for development and progression for breast cancer, due to the similarities of human mammary lesions.

We have completed the information in "Discussion". Please see manuscript.
4. The paper describes the incidence (rather than the presence) of CCLs in canine mammary gland specimens.

This work was based on the review of 126 cases with previous diagnosis of epithelial hyperplasia, it is not possible to estimate the prevalence of columnar lesions. In order to estimate the real prevalence of tumoral lesions, a morphological review of all cases attended in the Veterinary Teaching Hospital in a period of 10 years will be done (approximately 1500 cases), which consists on a future study of our group.

Material and Methods:
1. Please specify the lesions associated with CCLs.
   Ok. Modified. Please see manuscript.

2. The abbreviation for Columnar Cell Lesions without atypia would be CCLs not CCC because CCC stands for Columnar Cell Change. FEA includes both columnar cell change (not lesion) with atypia and columnar cell hyperplasia with atypia.
   Ok. Modified. Please see manuscript.

3. None of the antibodies seem to react with canine tissue. Authors should explain how the immunohistochemistry protocols were optimized. What were the positive and negative control tissues?
   Previous studies demonstrate the antibody reaction on canine mammary tissues. However, we used human breast cancer tissue as positive control, which presents reliable expression of the antibodies. Negative controls were assessed using normal serum as the primary antibody.
   We have completed the information. Please see manuscript.

4. Please specify exactly which visualization system was used.
   The immunohistochemical expression was analyzed using the Leica-Qwin (Cambridge, UK) image system.
   We have completed the information. Please see manuscript.

5. Since CCLs are not invasive tumor specimens, it is not clear why it is reasonable to use ASCO/CAP guidelines for HER2 scoring. Furthermore, none of the samples were HER2-positive, so HER2 expression could not be scored according to these guidelines.
The classification ASCO/CAP was proposed as a score method only, however, no type of superexpression was identified. We decided to keep this classification and make clear that a method of Her2 expression analysis was performed.

6. Were these canine tissues selected for mammary lesions (invasive tumors or other lesions?) when the specimens were first collected? In another words, it is important to know whether these CCLs were tumor-associated lesions or randomly sampled mammary tissues. According to Table 1, only 7 samples had no associated tumor.

The 126 mammary glands samples were obtained from the Veterinary Teaching Hospital of the Federal University of Minas Gerais, after clinical diagnosis of mammary tumor and surgical removal of the lesion. We have completed the information. Please see manuscript.

Results:
1. According to Para , associated lesions were represented only by ductal and lobular hyperplasias but Table 1 includes invasive malignant tumors, in situ carcinomas, and benign tumors. Were CCLs associated with both ductal hyperplasia and invasive, in situ or benign tumors? This should be clarified.

Ok. Modified. Please see manuscript.

2. What were the associated benign and malignant tumors?
Ok. Modified. Please see manuscript (table 1 and 2)

3. What does ‘apically dilated acini’ mean?
Ok. Modified. Please see manuscript.

4. If five cases of columnar cell change (CCC) showed more than two cell layers, these could not be classified as CCC, because by definition CCC should have one to two layers of columnar epithelial cells. The authors were correct when they classified these 5 cases as CCH without atypia. However, the term columnar cell lesion (CCL) should be used instead of CCC. Ok. Modified. Please see manuscript.

5. Were 34#E-12, p53 and HER2 all negative in invasive tumors?

The cases were reviewed and reported in the article.
Figures
Fig. 1. According to the figure legend, 1A shows normal mammary acini. Are not all four figures CCCs (1B definitely is a high-power view of the upper right area of 1A)? ‘Normal mammary acini with dilated lumina’ should be changed to ‘dilated mammary acini’ as TDLUs are not normal in CCL.

Ok. Modified. Please see manuscript.

Fig. 3. Acini lined with 1-2 cell layers do not show cytological atypia. Different CCL subtypes may coexist in the same breast. Therefore, the figure legend should be focused on atypical features.

Ok. Modified.

Fig. 6. There is high background staining with ER and moderate background staining with Ki67. Membranous staining is difficult to appreciate with E-cadherin. Please provide higher resolution figures.

Ok. Modified.

Discussion:
1. Was there any association between CCLs and pregnancy?

It was not possible to make this evaluation in the present work.

2. Myoepithelial/basal and some epithelial cells in human CCLs are positive for CK5/6 (Mod Pathol 2008;21:1413-20). Also, according to the antibody insert sheet, 34#E12 stains all epithelial layers including luminal and basal epithelium and ductal cells in human breast (Mod Pathol 1997; 10:93A). In our experience, wild-type p53 is also weakly expressed in human CCLs.

The informations of the cited references were inserted in the present article.

3. In human breast, CCLs have been described under a variety of terms including blunt duct adenosis, columnar alteration with prominent apical snouts and secretions, columnar metaplasia, enlarged lobular units with columnar alteration (AJSP 2005;29:105). Have similar lesions been described in dogs? Are there any synonymous terms?

No similar description or synonymous terms was found in the consulted literature. According to the veterinarian classification, adenosis consists of non-neoplastic proliferation of ductules, without cellular atipias. This lesion does not fit in CCLs classification.

4. What treatment modalities may be tested on canine tissues for CCLs?

A future study will be proposed for molecular evaluation of these lesions in the female dog, using hybridization techniques and tissue microarray. However, this is not the objective of our work.

5. Please state the limitations of the work such as antibodies not tested on
The markers used in the present work are well established in the veterinary literature for characterizing neoplastic and non neoplastic mammary lesions.


6. More references should be acknowledged.
Ok. Modified. More references were added, please see manuscript.

7. The paper needs some language corrections, for example, ‘They were observed in the terminal duct lobular units and characterized at dilated acini lined with a single layer of columnar epithelial cells with elongated nuclei’ in Abstract; ‘Flat epithelial atypia presence is often associated with in situ carcinomas…’ in Figure 4 legend, etc.

Figures 4 and 5 were eliminated. Please see Table 1 and 2.

- Minor Essential Revisions
1. Figure 4: Correct the title ‘…whith malignant e bening tumors’ and the label on the chart ‘w hitout tumor’

Figures 4 and 5 were eliminated. Please see Table 1 and 2.

2. UDH stands for usual ductal hyperplasia. Please add it to the abbreviation list.
Ok. Modified.

3. Abbreviation is misspelled.
Ok. Modified.