Author's response to reviews

Title: The dog as a naturally-occurring model for insulin-like growth factor type 1 receptor-overexpressing breast cancer: an observational cohort study

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Version: 2 Date: 6 August 2015

Author's response to reviews: see over
First, the authors would like to thank the reviewers for the time dedicated in reviewing our manuscript. We totally agree with most of the reviewers’ comments. All the inconsistencies and mistakes noted by the three reviewers have been rectified. All the corrections are highlighted in yellow within the revised manuscript. We tried to provide answers as completely as possible and arguments when needed for each reviewer’s comment.

Reviewer's report
Title: The dog as a naturally-occurring model for insulin-like growth factor type 1 receptor-overexpressing breast cancer: an observational cohort study
Version: 1. Date: 6 July 2015
Reviewer: Lorella Maniscalco

Reviewer's report:
The manuscript entitled "The dog as a naturally-occurring model for insulin-like growth factor type 1 receptor-overexpressing breast cancer: an observational cohort study" is a well written and very interesting study but there are several major consideration regarding the inclusion criteria of the cases.
This study considers a retrospective selection of canine mammary tumor and considers disease free interval and overall survival basing on telephone interview with veterinarians. I suggest the author to include in this study cases with this inclusion criteria because in this present form, each consideration regarding the prognostic value of IGF1R cannot be accepted.

It is the authors’ opinion that the prognostic value of IGF1R expression can be assessed even if the method of follow-up is based on telephone interviews. The dogs included in this series represent only a proportion of dogs diagnosed with invasive mammary carcinoma in the study period, i.e., the dogs for which sufficient follow-up data could be collected. Dogs were included if it was apparent from their medical history that regular presentations to the veterinary practitioner allowed identifying locoregional recurrence, distant metastasis, and/or intercurrent disease.

- all the patients with similar clinical conditions at the time of surgery (i.e tumor with radiographically and or TC negative for metastasis)
  It was the authors’ choice not to exclude stage V carcinomas from the study (i.e., cases presented with distant metastases, N=4).

- same surgical treatment (total or partial mastectomy with free surgical margins)
  It was the authors’ choice not to exclude dogs treated with incomplete surgery, at least to be able to identify the prognostic value of involved surgical margins (N=82), as appears in Tables 3, 4, and 5.
  We have voluntary chosen to gather the largest cohort as possible, even if pieces of information were sometimes missing and clinical presentations were not uniform, in order to be as representative as possible of the natural history of dogs with invasive mammary carcinoma. In veterinary medicine, there are no consensus statements on the management of dogs suffering from mammary carcinomas. For instance, total mastectomy is not systematically performed and depends on the veterinarian and the owner’s preference. In the cohort studied, 67 dogs had a total mastectomy, 56 a half-chain mastectomy, 18 a single-gland mastectomy and 9 a lumpectomy. Lumpectomy could be considered as equivalent of “breast-conserving surgery” in humans, and does not constitute an exclusion criterion in retrospective studies of breast cancer in humans.
clinical controls performed every 3-6 months with at least a radiographic study.

In daily veterinary practice in France, X-ray and/or ultrasound are not systematically performed at the time of diagnosis of a mammary tumor in a dog, but are more likely to be performed in the follow-up period of a dog diagnosed with invasive mammary carcinoma. This is the reason why stage V (M1) cases (N=4) are likely underestimated in the cohort presented, whereas distant metastases were quite common at the end of follow-up (N=45). However, X-ray and ultrasound are not sensitive enough to detect early metastasis and all veterinarians do not have access to more sensitive detection methods. Thus, distant metastases were also very likely underestimated at the end of follow-up. This understage of canine mammary carcinoma will probably hold true for some more years, as very few French dogs are insured (unlike in other countries as USA or Great Britain) and most owners cannot afford additional diagnostic tests.

The aim of the study was to describe IGF1R expression in a large cohort of dogs with invasive mammary carcinoma with special emphasis on its prognostic value. We think that the results of this study are valuable despite the retrospective design of the study. Unfortunately, selection and information bias are unavoidable parts of retrospective studies. If the reviewer thinks that the discussion needs to be supported with the advantages and disadvantages of the retrospective aspect of this study, we could add such a paragraph in the discussion.

The author used improper terminology regarding the survival study. Please used the conventional term OS with censored and not censored cases. Specific survival is not a conventionally used term in survival studies.

Specific survival was defined as the period of time between the histological diagnosis of the invasive mammary carcinoma and cancer-related death, as opposed to overall survival that was defined as the time between histological diagnosis and death from any cause. This terminology is conventional and appropriate (Hudis, Barlow et al., J Clin Oncol 2007, 25: 2127-2132. Dignam, Huang et al., Cancer 2009, 115: 5272-5283).

In addition, some human studies on IGF1R in breast cancer used breast cancer specific survival analysis in their survival study (for instance, Hartog et al., Breast Cancer Res Treat 2011, 129:725-736; Law et al., Cancer Res 2008, 68:10238-46).

We slightly modified the sentences regarding overall and specific survival, pages 5-6, lines 125-131, in order to explain censures in greater detail.

As another reviewer pointed out, specific survival was more appropriate than overall survival to analyze the real prognostic value of IGF1R expression. In dogs, unlike humans, death can be due to many other causes than the invasive mammary carcinoma, and is in particular biased by euthanasia for non-medical purposes. That is the reason why we think that specific survival analysis is more informative than overall survival in this study.

- The author states "The interval from surgery to the first local recurrence. New primary tumor.." what do the author evaluate to consider a new primary tumor as recurrence of a previous one and not as a new one? (page 6, lines 131-132) A new mammary tumor was considered as local recurrence of the invasive carcinoma if it developed exactly at the same place as the invasive carcinoma (i.e., the same mammary gland). A new mammary tumor that developed in another mammary gland than the invasive carcinoma was defined as a new
primary tumor.

Minor revision:
- line 30: the definition of TN is "lack of ER, PR and HER2 overexpression". We have changed HER2 expression by HER2 overexpression.

- line 59: different author in literature consider canine mammary tumor not such a good model. Please complete the background including for what is considered a good model or not a good model. Feline is a better model for human breast cancer, especially for TN one. We agree with the reviewer’s comment that feline mammary carcinoma is a better model for human triple negative breast cancer than canine mammary carcinoma, although this is not the purpose of this study to discuss the feline model. As far as IGF1R and other obesity-related markers are concerned, only dogs – not cats – are valuable models for human breast cancer, as their diet and propensity to overweight is far closest to humans than cats.

It is correct that some histological entities of mammary carcinomas are quite different between human and dog. We added the following sentence (lines 64-65): “even if some histological entities (particularly complex mammary carcinoma) are quite different between human and dog [6]”. However, in the authors’ experience, most complex mammary carcinomas of dogs fall into the category of ductal carcinomas in situ, and very few of them are invasive. That is the reason why the cohort studied comprised only 7% complex carcinomas. In the authors’ opinion, canine mammary carcinomas are excellent spontaneous models of human breast cancer, provided that carcinomas in situ are excluded, as was the case in this study.

- 133: why the cases were also evaluated by a human pathologist? Do they were in agreement or not? The evaluation by a human pathologist was critical in this study; Dr Delphine Loussoarn performed all of the HER2 scorings with the same method as she uses in breast cancers. For all other histological and immunohistochemical data, the results presented are the consensus found among the 5 pathologists (1 human, 4 veterinarians). The degree of agreement between pathologists was moderate at the beginning of the study, but increased significantly as the study progressed.

- 154-155: I suppose that author used the Allred method of evaluation use by several authors also in feline and canine mammary tumors. Please use a different reference. No, the authors did not use the Allred scoring system for ER and PR evaluation; we measured the ER and PR indexes on at least 500 nuclei of neoplastic cells, with the help of the software imageJ for image analysis. In this method, the index is calculated by taking into account any positive nucleus whatever its staining intensity. As a consequence of using an index, ER and PR are continuous variables (percentages), as opposed to the Allred score, which is an eight-scale discrete variable. Thus, ER and PR are evaluated as positive or negative using a threshold, defined at 10% in this study.

- 168-170: cite Peña et al 2014 or Wolff et al 2007. Now line 174 in the revised manuscript. We added the reference (Wolff, Hammond et al., J Clin Oncol 2013, 31: 3997-4013) [34].

Level of interest: An article whose findings are important to those with closely related research interests.
Quality of written English: Acceptable
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.
Declaration of competing interests:
I declare that I have no competing interests' below
Reviewer's report
Title: The dog as a naturally-occurring model for insulin-like growth factor type 1 receptor-overexpressing breast cancer: an observational cohort study
Version: 1. Date: 5 July 2015
Reviewer: Josef Singer

The article 'The dog as a naturally-occurring model for insulin-like growth factor type 1 receptor-overexpressing breast cancer: an observational cohort study by Laetitia Jaillardon, Jérôme Abadie, Tiffanie Godard, Delphine Loussouarn, Mario Campone, Brigitte Siliart and Frédérique Nguyen is a well-elaborated and concise observational cohort study of IGF1R expression in canine mammary carcinoma within the context of Comparative Oncology. 150 patients were included in the cohort and monitored for 2 years with respect to disease-free interval, overall survival and specific survival.

The size of the cohort is appropriate and their main experimental method, immunohistochemistry, is well performed and in the supplementary information also well described. As many clinical oncologists are used to the HercepTest™ classification, it is suitable to score also IGF1R expression analogous to this scoring system. Fig. 1 nicely depicts the different scoring intensities, and it is creditable that only 3+ lesions were considered as IGF1R positive.

Another positive aspect of this study, which should be highlighted, is that each specimen was classified by five independent pathologists.

A rather astonishing finding of this study is, that the authors could not find any HER-2 overexpressing carcinoma in 150 patients. As this is against other published studies, it is interesting whether this finding is real or a methodological issue. In human medicine and in several veterinary oncology studies, pathologists use the HercepTest™ to evaluate HER-2 expression. As this was not used in this study, but the authors reference to the scoring system, I would suggest to stain the sections also with this staining kit. The side-by-side comparison of the two different staining systems (Monoclonal rabbit anti-human Clone 4B5 vs. Polyclonal rabbit HercepTest™) will be also interesting for other researchers working in the field.

Please refer to our answer on the next page.

The results of the study are well-elaborated, the prognostic value of IGF1R expression in canine mammary carcinoma is well-investigated, especially within the important context of luminal subtype or triple negative mammary carcinoma.

These findings are also put in line with current literature and the similarities and differences between human studies are discussed, which is of utmost importance for Comparative Oncology. However, only for the human situation possible mechanistic explanations are discussed by mentioning in vitro studies investigating the crosstalk between the IGF1R and ER. As this study shows, that both subtypes of mammary carcinoma, luminal and triple negative, have worse prognosis upon IGF1R overexpression in dogs, it would be interesting to know, whether eventual crosstalk between the IGF1R and the ER have also been investigated in canine tumor cells. If not, please indicate in the paper, that here possible differences within the receptor biology can be expected and should thus be further investigated.

To our knowledge, no in vitro study to date has investigated the crosstalk between IGF1R and ER in canine mammary tumor cells. However Queiroga et al. (2008) [20] showed that IGF1
levels in tissue homogenates were positively associated with 17beta-estradiol levels and Dolka et al. (2011) [21] showed that IGF1R expression was positively correlated with ER expression (Dolka 2011), however in small cohorts of dogs (respectively 40 and 47 malignant neoplasias, without specification of their invasive or in situ mode of growth).

In accordance with the reviewer’s comments, we added this sentence (lines 349-352): “However, no study to date has investigated the crosstalk between IGF1R and ER in canine mammary cell lines. A difference of receptor biology between Human and Dog cannot be excluded and should thus be further investigated”. In addition, concerning the bad prognostic value in both the luminal and the triple negative subtypes, the authors cannot exclude an interaction with the treatment in ER positive breast cancer. As a result, we add the following sentence (lines 364-367): “The fact that none of the dogs of this study received adjuvant endocrine therapy is however a major difference between humans and dogs after a diagnosis of luminal mammary carcinoma, and this difference is likely to interfere with prognosis. Furthermore, only 47 luminal mammary carcinomas were included in this study and further investigations with a higher number of luminal mammary carcinomas are needed to confirm this result.

Apart from that, the discussion is clear and informative, the structure of the paper is well designed to navigate the reader through the experiments and the quality of written English is excellent.

Summarizing, this manuscript is of good quality and an article of importance not only for veterinary oncology but within the context of Comparative Medicine also for human clinical

Major compulsory revisions:
Please stain again for HER-2 with the HercepTest™ kit, as it is highly unlikely to have not a single positive tumor overexpressing HER-2 in 150 mammary carcinoma patients.
All of the cases included in this series have been immunostained with both the 4B5 rabbit monoclonal antibody from Roche Diagnostics, and the HercepTest™ kit using the rabbit polyclonal antibody from Dako. It was the authors’ choice to present only the results obtained with the 4B5 antibody in this publication. With both anti-HER2 antibodies, the cohort did not comprise any HER2-positive case (score 3+). The level of agreement between the 2 anti-HER2 antibodies was as follow:

<table>
<thead>
<tr>
<th>HercepTestTM score</th>
<th>4B5 score 0</th>
<th>4B5 score 1+</th>
<th>4B5 score 2+</th>
<th>4B5 score 3+</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>77</td>
<td>22</td>
<td></td>
<td></td>
<td>99</td>
</tr>
<tr>
<td>1+</td>
<td>8</td>
<td>24</td>
<td>5</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>2+</td>
<td>4</td>
<td>10</td>
<td></td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>3+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>85</td>
<td>50</td>
<td>15</td>
<td>0</td>
<td>150</td>
</tr>
</tbody>
</table>

111/150 cases (74%) had the same immunohistochemical score with the 4B5 antibody and the HercepTest kit.

As this finding would be against many published studies, this has to be investigated in more depth.
We agree that our finding is in disagreement with previous published data on HER2 immunohistochemical expression in canine mammary carcinomas. Among the possible explanations for this discrepancy, we can cite:
- the fact that we use short incubation times for the primary antibody (8 min for the 4B5 clone), compared to previously published reports (30 min, 3 hours, or overnight incubation times), although this short incubation time is compensated by the use of a
very sensitive detection system (Ultraview DAB detection system for automated
immunohistochemistry)
- the fact that we set up the immunohistochemical protocols for HER2 detection using
commercially available control slides (Pathway Her2 4-in-1 control slides, Roche
Diagnostics), which contain 4 spots of human breast cancer cells, corresponding to
scores 0, 1+, 2+ and 3+. The use of this appropriate control was critical in order to set
up a protocol for HER2 detection with the 4B5 clone, which does not overstain the 2+
cases.

Moreover, HER-2 status is part of the classification as triple negative, therefore it is important
to exclude methodological issues. All positive controls for HER-2 of this study are expressing
human HER-2, so maybe there is a lack of cross-reactivity of the monoclonal antibody 4B5 to
the canine counterpart.
The cross-reactivity of the monoclonal antibody 4B5 to canine HER2 was checked at Roche
Diagnostics (Penzberg, Germany) before the beginning of the study. It is the authors’ choice
not to include these results in the present publication.

For the polyclonal HercepTest™, cross-reactivity could already be demonstrated.
It has been challenged recently by Burrai et al. (Tumor Biol 2015, PMID: 26088453), who
suggest a lack of specificity of the polyclonal antibody from Dako in canine mammary tumor
samples.

The significance of HER2 overexpression in canine mammary carcinoma is unclear.
Controversial results exist and are not consensual. Sassi et al. (BMC Veterinary Research
2010, 6:5) found 22/45 HER2-positive canine mammary carcinomas using the polyclonal
Herceptest™ kit, but their definition of HER2 positivity was “when at least 10% tumour
cells showed moderate to strong complete membranous staining”, a definition that encompasses
scores 2+ and 3+.
HER2 overexpression in canine mammary carcinomas is rare in other reports. Gama et al.
(Virchows Arch 2008, 453:123-132) found only 8/100 HER2-overexpressing canine
mammary carcinomas with another monoclonal antibody (clone NCL-CB11, Novocastra).
Although Martin de las Mulas et al. (Breast Cancer Res Treat 2003, 80: 363-367) found that
18% of canine mammary carcinomas overexpressed HER2, using the polyclonal Herceptest™
kit, their cohort did not comprise any positive case by chromogenic in situ hybridization
(CISH).
A recent study by Burrai et al. (Investigation of HER2 expression in canine mammary tumors
by antibody-based, transcriptomic and mass spectrometry analysis: is the dog a suitable
animal model for human breast cancer? Tumor Biology 2015) suggests a lack of specificity
of the FDA-approved antibody Herceptest™ in canine mammary tumor samples as
demonstrated by Western immunoblotting, reverse phase protein arrays and mass
spectrometry. In this study, HER2 overexpression by immunohistochemistry (score 3+) was
observed in 2/9 canine mammary carcinomas, but “a diffuse non-specific cytoplasmic staining
pattern was observed in most of the immunoassayed lesions”. By Western Blot, “no signal
could be observed in all canine mammary samples at the expected MW of 138 kDa, whereas
two non-specific bands could be detected at lower MWs (between 50 and 60 kDa) in almost
all samples”. By mass spectrometry, “no HER2 (or other HER family) peptides could be
detected neither in the higher MW bands from the canine mammary samples nor in the lower
MW bands”.

To summarize, we are aware of the discrepancy between our cohort (total absence of HER2-
overexpressing canine mammary carcinoma) and previously published cohorts (18-30% of HER2-overexpressing cases). However, the absence of HER2-overexpressing case is in agreement with the reported absence of HER2 amplification in canine mammary carcinomas (Martin de las Mulas et al., 2003). The choice to present the results obtained with the 4B5 antibody only does not interfere with the finding that no case overexpressed HER2, as both antibodies were concordant in this respect, in the cohort presented.

Minor essential revisions:
In the methods section of the abstract:
Please change the last sentence to 'The prognostic value of the IGF1R expression was assessed in terms of overall AND specific SURVIVAL AS WELL AS disease-free interval (DFI).’ to make it easier to grasp for the reader.
The change has been made (line 39).

Please change the sentence from:
Anyway, the expression and prognostic value of IGF1R overexpression is of particular interest in the triple negative subtype since it is associated with a poor prognosis, particularly in young women for which this type is more frequent.
To:
NONETHELESS, the expression...
The change has been made (line 367).

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.
Declaration of competing interests:
I declare that I have no competing interests
Reviewer's report
Title: The dog as a naturally-occurring model for insulin-like growth factor type 1 receptor-overexpressing breast cancer: an observational cohort study
Version:1. Date: 1 June 2015
Reviewer: Patricia Dias Pereira

Reviewer's report:
The dog as a naturally-occurring model for insulin-like growth factor type 1 receptor-overexpressing breast cancer: an observational cohort study
This is an interesting and well-written manuscript focusing on IGF1R immunohistochemical expression in a series of canine mammary carcinomas.
The authors also assess the prognostic value of IGF1R immunoexpression.
There are, however, several points that require attention/clarification:

Minor Essential Revisions
Fig 1a does not seem to represent normal mammary gland tissue; it reminds me of hair follicles instead.
It was a mistake; of course this is a hair follicle. It has been corrected in the caption of figure 1 (lines 626-627).

Line 67 – reference 7 does not support the author’s statement; in fact, Kim and colleagues found only 18.7% of triple-negative cases in a large cohort (n=241) of canine mammary carcinomas. “and because the triple negative (TN) immunophenotype, one of the most aggressive breast cancer subtypes defined by the lack of ER (Estrogen Receptor), PR (Progesterone Receptor) and HER2 (Epidermal Growth factor Receptor type 2) expression, is predominant in dogs [6, 7].” “Predominant” was replaced by “well-recognized”.

Lines 97-98 and 281-282 – the role of IGF1R in canine mammary carcinogenesis is not discussed in this manuscript. To our knowledge, no study to date has investigated the specific role of IGF1R in canine mammary carcinogenesis. In canine mammary tumors, the existing studies on IGF1R are only observational and clinical studies. For example, Dolka et al. (2011) showed that higher proliferation was observed in tumors with medium and high IGF1R expression. In addition, the role of IGF1R in carcinogenesis was investigated in canine melanoma cells (Thamm et al., 2010) and osteosarcoma cells (MacEwen et al., 2004) (mentioned on lines 93-94).

Line 135 - Ref Elston and Ellis (1991) is missing from the references list. The reference has been added to the reference list ([31]).

Ref 29 does not constitute the official WHO classification system for canine mammary tumors. We agree with this comment but former Ref 28 (now Ref 29, Misdrop W, Else RW, Hellmen E LT: Histological classification of mammary tumors of the dog and the cat. In World Health Organization International Histological Classification of Tumors of Domestic Animals. 2nd edition. Edited by Armed Forces Institute of Pathology. Washington DC; 1999:1–59) is the official classification. We used former Ref 29 (now Ref 30) because this article provides a brief overview of the two histologic classification systems (the first classification system in 1974 and a modification in 1999 by Misdrop et al.) and also compares the grading systems for canine mammary carcinomas and their use for prognosis, along with the histologic classification.
On what basis did the authors classify canine mammary carcinomas into luminal and triple-negative subtypes? This is not explicit in the text and is not supported by bibliographic references in canine species.

Canine mammary carcinoma were classified as Luminal if ER\textalpha\ expression and/or PR expression was/were positive, i.e., if the ER index and/or PR index was/were $\geq 10\%$. Otherwise, the mammary carcinoma was classified as triple negative (ER\textalpha<10\%, PR<10\%, HER2 score other than 3+). This was mentioned on former lines 198-204 (now lines 205-208). We added another sentence (lines 204-205: “The 150 invasive carcinomas were classified as luminal and triple-negative according to ER, PR and HER2 expressions”). However, if the reviewer prefers a more explicit sentence, please do not hesitate to let us know. We added the references (line 205) that support such a classification in dogs (Sassi et al., 2010 [5]; Gama et al., 2008 [4]).

Line 209 –“date of diagnosis”: do the authors mean “date of surgery”? In some cases, the exact date of surgery was imprecise. That is why we decided to take the date of histological diagnosis as the reference for all cases.

The authors do not interpret and do not present reasonable biological explanations for the inverse association they observed between IGF1R and ER expression, nor for the correlation found between IGF1R overexpression and aggressive clinicopathological features. The authors restrict their discussion to the presentation of data obtained previously by others which is, in my opinion, insufficient.

We did not have any explanation about the inverse association observed between IGF1R and ER expression, as no study to date has been conducted in dogs to put in relief this IGF1R/ER crosstalk in canine mammary cell lines.

As the second reviewer asked, we added the following sentences:
- lines 324-329: “This contradictory result could be due to a biological difference concerning IGF1R and ER between dogs and humans. The fact that IGF1R parallels ER expression in canine mammary carcinoma in the study of Queiroga is also contradictory: however, the cohort was small (40 mammary carcinomas) and unlike the present study, the invasive nature of the mammary carcinomas was not assessed.”
- lines 349-352: “However, no study to date has investigated the crosstalk between IGF1R and ER in canine mammary cell lines. A difference of receptor biology between Human and Dog cannot be excluded and should thus be further investigated”.

Regarding the correlation between IGF1R overexpression and aggressive clinicopathological features, we gave some introductory statements on former lines 68-72 (now lines 70-74: “In various human cancers including breast cancer, the Insulin-like Growth Factor (IGF) family is closely related to oncogenesis [8, 9], in situ tumor growth [10], invasion and metastasis [10], with IGF1R (Insulin-like Growth Factor Type I-Receptor) acting as a real oncogene and being overexpressed in more than 50% of primary breast cancers [11]”). We added the following sentence in the discussion (lines 338-340): “This finding is in line with the fact that IGF1R is considered as a real oncogene closely involved in survival, proliferation, tumor growth, invasion and metastasis as it was demonstrated in canine osteosarcoma-derived cell lines [23].”

**Discretionary Revisions**

IGF1R immunoeexpression is not well characterized in canine normal mammary tissue. In fact, the authors describe that “IGF1R was strongly expressed in the normal mammary tissue...
adjacent to the tumors”, while ref 21 documents” weak to moderate IGF1R expression in canine normal mammary gland”, similarly to data obtained by Bhargava and colleagues (ref 39) in human breast tissue. The authors do not provide a rational explanation for this finding. It would be very interesting to include samples of normal mammary gland (obtained from animals free of neoplastic disease) instead of considering the mammary tissue surrounding the tumour as normal. IGF1R lower- or over-expression should be defined considering the immunoreactivity of the normal mammary tissue as reference.

We totally agree with the reviewer comments about IGF1R expression in normal mammary gland of healthy dogs. Actually, we performed immunohistochemistry with anti-IGF1R antibody in normal canine mammary gland and we found moderate IGF1R expression, defined as complete and weak to moderate membrane staining in more than 10% of the cells.

In accordance with the reviewer’s comment, we have changed the sentence “IGF1R was strongly expressed in epithelial cells of the hair follicles, normal and hyperplastic mammary tissues adjacent to the tumors (Figure 1)” (lines 224-225) into “IGF1R was strongly expressed in epithelial cells of the hair follicles, hyperplastic and dysplastic mammary tissues adjacent to the tumors (Figure 1)”.

Rather than scoring IGF1R with the normal mammary tissue as reference, we have chosen to score IGF1R expression according to the HER2 expression scoring system, in accordance with Shin et al. (2014) [19] and Shimizu et al. (2004) [35] to facilitate comparisons with breast cancers.

The authors do not explain why they decided to stratify tumor size into <2 and >2cm categories and body size into >10 and <10 Kgs.

The tumor size categories were chosen because 20 mm is the threshold between pT1 and pT2 breast cancers according to the American Joint Committee on Cancer (AJCC). We are aware that the thresholds for tumor size in the canine species are 3 and 5 cm; however, these thresholds correspond to clinically measured mammary carcinomas. In the present study, tumor sizes were determined by the pathologists, and correspond to “pathologic tumor sizes” (pT). The following sentence was added to the manuscript (Lines 143-144) “The histologically assessed size of mammary carcinoma(s) with 2 cm chosen as a threshold according to the American Joint Committee on Cancer (AJCC)”

The body size categories (<10kg, 11-25 kg and >26kg) were chosen according to previous results of our team, about IGF1 reference values in healthy dogs and dogs with primary hypothyroidism (Jaillardon et al., Serum insulin-like growth factor type 1 concentrations in healthy dogs and with spontaneous primary hypothyroidism. Vet J. 2011. 190(2):e95-99). Indeed, we found that serum IGF1 was highly dependent on the canine standard breed body weight. Actually, in this previous study four categories were used (<15kg, 16-25 kg, 26-40kg and >41 kg) but a category of less than 10 kg was better in terms of representativeness and prognostic value in the present study, partly due to the high number of small breed dogs in the cohort.

Despite the low number of carcinomas scored 0 for IGF1R expression, it would be interesting to know if those cases share similar clinicopathological features. Unfortunately, there was no uniform presentation for the carcinomas scored 0 as shown in the table below shows:

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age category</td>
<td></td>
</tr>
<tr>
<td>Category</td>
<td>Count</td>
</tr>
<tr>
<td>---------------------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>3-10.9 yrs</td>
<td>4</td>
</tr>
<tr>
<td>&gt;11 yrs</td>
<td>7</td>
</tr>
<tr>
<td>Body size</td>
<td></td>
</tr>
<tr>
<td>&lt;10 kg</td>
<td>3</td>
</tr>
<tr>
<td>11-25 kg</td>
<td>4</td>
</tr>
<tr>
<td>&gt;26 kg</td>
<td>1</td>
</tr>
<tr>
<td>Carcinoma size</td>
<td></td>
</tr>
<tr>
<td>&lt; 2 cm</td>
<td>6</td>
</tr>
<tr>
<td>&gt; 2 cm</td>
<td>5</td>
</tr>
<tr>
<td>Central necrosis</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>8</td>
</tr>
<tr>
<td>No</td>
<td>3</td>
</tr>
<tr>
<td>Complete excision</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>5</td>
</tr>
<tr>
<td>No</td>
<td>6</td>
</tr>
<tr>
<td>Cutaneous ulceration</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>2</td>
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<tr>
<td>No</td>
<td>9</td>
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<tr>
<td>Distant metastasis</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
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Major Compulsory Revisions
I have serious reservations regarding the follow-up study:
- Phone call (to the owners or to the clinicians) is an unreliable follow-up method.
Animals included in this study were not submitted to standardized periodical clinical examination, as recently recommended by Matos et al (2012), which may bias some of the clinicopathological features evaluated in this investigation namely date and histological confirmation of local recurrence, development of new tumoral lesions, lymph node metastasis and/or distant metastasis.

We totally agree with the reviewer’s statement about the follow-up method. Unfortunately, this was a retrospective study and the majority of the dogs were already dead at the time of the collection of the data. As we argued in our reply to the first reviewer’s comments, the retrospective design of the study implies a likely bias of underestimation of post-surgical events (recurrence, metastasis).

It was our concern however that the dogs included in this study had the most complete medical history as possible, with regular presentations to their veterinary practitioner after the diagnosis of invasive mammary carcinoma. Dealing with mammary tumors that are externally located and thus palpable and/or visible, the risk of underestimation of locoregional recurrences seems low, as owners and veterinarians are aware of the importance of locoregional control.

The aim of the study was to describe IGF1R expression in a large cohort of dogs with invasive mammary carcinoma with special emphasis on its possible prognostic value. We think that the results of this study are informative despite the retrospective design of the study. Unfortunately, selection and information bias are unavoidable in retrospective studies. If the reviewer thinks that the discussion needs to be supported with the advantages and disadvantages of the retrospective aspect of this study, we could add such a paragraph in the discussion, pointing out the limits of this study.

- Furthermore, I don’t find “overall survival” (as defined by the authors) a useful prognostic parameter as it includes death from any cause, that may not be related to the tumoral lesion. I suggest eliminating this prognostic indicator.

We agree that overall survival may be less appropriate than specific survival to assess the prognostic value of a parameter in the canine species. Indeed, some dogs may be euthanized because of the economic burden of disease rather than for medical reasons. However, the present study was designed with comparative oncology in mind, and overall survival is by far more often studied than cancer-specific survival in survival studies of breast cancer. Thus, both overall and specific survival were presented in the manuscript.

Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests:
I declare that I have no competing interests
The authors hope that these revisions will be satisfactory to the reviewers and will respond with enough precision to their requests. Please, do not hesitate to let us know if you think that some of the previous comments and answers are lacking in the manuscript and have to be added.