Author's response to reviews

Title: Estrogen receptor alpha/beta ratio and estrogen receptor beta as predictors of endocrine therapy responsiveness - a randomized neoadjuvant trial comparison between anastrozole and tamoxifen for the treatment of postmenopausal breast cancer

Authors:

Marcelo Madeira (marcemadeira@gmail.com)
Andre Mattar (mattar.andre@gmail.com)
Angela F Logullo (waitzberg.angela@gmail.com)
Fernando A Soares (fasoares@me.com)
Luiz H Gebrim (lgebrim@uol.com.br)

Version: 2 Date: 31 July 2013

Author's response to reviews: see over
Author's response to reviews

Original Title: Estrogen Receptor Beta as a Predictor of Endocrine Therapy Responsiveness – A Randomized Neoadjuvant Trial Comparison Between Anastrozole and Tamoxifen for the Treatment of Postmenopausal Breast Cancer

Authors:

Marcelo Madeira (marcemadeira@gmail.com)
André Mattar (mattar.andre@gmail.com)
Ângela Flávia Logullo (waitzberg.angela@gmail.com)
Fernando Augusto Soares (fasoares@me.com)
Luiz Henrique Gebrim (lgebrim@uol.com.br)

Version: 2 Date: 28 July 2013

Author's response to reviews: see over
São Paulo, July 28, 2013

Editor-in-Chief of *BMC Cancer* and The Biomed Central Editorial Team

**Object: MS: 1462211937968627** - Estrogen Receptor Beta as a Predictor of Endocrine Therapy Responsiveness – A Randomized Neoadjuvant Trial Comparison Between Anastrozole and Tamoxifen for the Treatment of Postmenopausal Breast Cancer

Thank you very much for your considerations of our manuscript for publication in *BMC Cancer*.

The authors are grateful and have reviewed the above manuscript according to reviewer’s valuable comments.

Best regards,

Marcelo Madeira
Reviewer # 1 (Dr Tesa Severson)

Reviewer's report
Version: 1 Date: 15 May 2013

Major Compulsory Revisions:

- Title: The title currently is misleading. The data support ER# along with ER# can predict response to endocrine therapy. The title needs to be altered to reflect the predictive power of the ER#/ER# ratio, rather than ER# alone.

  The title of the article has been changed as the reviewer indicates. As we studied ER-β-positive BC independently of ER-α status and these specific results from our trial demonstrated that ER-β-positive BC treated with anastrozole and tamoxifen presented a significant reduction in Ki67 expression compared with placebo and ER-β-negative cases, we have also maintained “estrogen receptor beta” in the title of the manuscript.

- Page 11, paragraph 1: The use of the Allred score for determination of IHC staining should be defended with a figure showing IHC assays are not over saturated. Otherwise, use the proportion of cells staining positive.

  We have added a figure (new Figure 2) showing Immunohistochemical staining pattern in BC samples. We included examples of ER-β (liver as negative control, whole section positive control slide and Allred scores), ER-α and Ki67 expressions.

  We haven’t submitted such figure before because of the consequent large file. We were afraid of it would be technically difficult to publish in print given the nature of most journal page formats.

  Thank you again for your valuable comments and suggestions.

- Page 13, paragraph 1: Since the claim is that more information can be gained with the addition of ER# staining, ER# data needs to be for added comparison.
As suggested by another reviewer, we have included a table (new table 2) indicating how many cases belong to each study group and the number of ER-α-positive in the different categories.

- Page 13, paragraph 2: Only 57 of 78 cases were ER# positive. In a clinical situation these patients would not typically be treated with endocrine therapy. Comment on these patients and their inclusion in the study.

  Probably the reviewer refers to the 21 (78 – 57) patients who had ER-α-negative breast cancer and were included in our study. That’s why a treatment period of 26 days was chosen. This is the average time needed to complete routine preoperative testing in our institutions, justifying the use of placebo without ill consequences to the ER-α-positive patients and the inclusion of ER-α-negative patients. We included some explanations about this topic and responding the comment 6 (tamoxifen dose) as a new second paragraph of the discussion.

- Page 13, paragraph 2: The title of the study is “Estrogen receptor beta as a predictor of endocrine therapy responsiveness – a randomized neoadjuvant trial comparison between anastrozole and tamoxifen for the treatment of postmenopausal breast cancer”. To conclude that ER# is a predictor of endocrine therapy response when the group, which is ER# positive and ER# negative, is too small for statistical analysis is premature. In fact, those patients with a ratio <1 of ER#/ER# (page 14, paragraph 1) were not significantly different in their Ki67 levels suggesting there may be little prediction value for endocrine therapy response in ER# only cases. I suggest changing the title of the paper to reflect the importance of the ratio of ER#/ER# or examine more ER# positive/ER# negative patients or retrospective assessment of ER# from other trials to determine the validity of the current title.

  The reviewer is correct and we have added the expression “*estrogen receptor alpha/beta ratio*” to the Title. Our aim is to conduct another study with a larger number of ER-α-negative patients to compare neoadjuvant treatment with AI and tamoxifen in ER-β-negative versus ER-β-positive cases.
Comment on the dose of tamoxifen, is 20mg/day enough without a loading dose to reach steady state in 26 days? In addition, these patients are compared with patients treated with AIs which reach steady state levels much faster than tamoxifen. Is there a bias in the data because of this? (Soininen et al. J. Int. Med. Res. 1986 and Buzdar Clin. Can. Res. 2003) Measurements from the plasma of patients showing therapeutic levels of tamoxifen after treatment would be useful.

We have added some explanations about the dose of tamoxifen to the Discussion (new second paragraph). The sentence in the manuscript now appears as:

"The optimum time to evaluate biomarkers for tumor response (apoptosis and proliferation) is not defined. Although cellular changes have been described in vitro after 24 hours of drug exposure, Dowsett et al (ref) reported that after two weeks of neoadjuvant treatment of primary breast cancer with anastrozole and tamoxifen, cellular changes are similar to those observed after 12 weeks of treatment. As other similar studies (refs), the classical dose of tamoxifen (20mg/day) is enough to reach steady state after 14 days of short-term treatment. The period of 26 days was chosen because this is the average time needed to complete routine preoperative testing in our institutions, justifying the inclusion of ER-α-negative patients and the use of placebo without ill consequences to the ER-α-positive patients".

Add a section where limitations of the work are clearly stated.

Some limitations were added in the manuscript (end of Discussion) as followed:

"Our study was hampered by relatively small sample size. The number of cases according to positive or negative hormone receptors (especially for the ER-β-negative and ER-α-negative cases) prevented a separate statistical analysis of Ki67 changes after treatment in each group. A systematic and larger study, taking ER-β status into consideration, for patients with different positivity for receptors (ER-β, ER-α and PgR) could better characterize each cancer and help to optimize adjuvant treatment for BC patients. Some differences of our conclusions compared with other studies should be drawn keeping in mind the large amount of ER-β antibodies used in the literature and the various cut points for determining the positivity of ER-α and ER-β."
Some published data on the usefulness of several ER-β antibodies for a number of analyses including immunohistochemistry have underscored the marked variations in specificity and likely sensitivity that exist for the different antibodies currently available (ref). In addition, our Brazilian population is one of the most heterogeneous in the world, formed mainly by the admixture between European, African and Native American populations and, more recently, individuals of Asian origins. These race-specific factors may also influence our findings compared with the white population of others studies. Although no studies have examined specifically differences in ER-β protein expression with regards to ethnicity, two studies showed that ER-β mRNA levels are significantly decreased in ER-α-positive BC from African American women (ref) and from East Asian women (ref). It should also be noted that the patients enrolled onto this trial represent only a small percentage of our whole postmenopausal BC population treated in our institutions during the entry period. Several studies failed to find significant correlations between ER-β expression and patient age (ref), however, it may be considered another limitation of our study.”

Minor Essential Revisions

- Page 1, paragraph 2, sentence 2: remove “final”.
  Done (We interpreted that the reviewer has named Page 1 as the Abstract).

- Page 1, paragraph 2, sentence 3: remove “to”.
  Done (We interpreted that the reviewer has named Page 1 as the Abstract).

- Page 6, paragraph 2, sentence 1 and 2: references required.

- Page 9, paragraph 1, sentence 1: What is the range for data described?
We added all the information that was available to address this comment as followed:

“The patients underwent definitive surgical treatment (modified radical mastectomy or conservative surgery with axillary evaluation) following a mean period of 26 days (range of 24-30 days and median of 26 days) after the incisional biopsy”.

- Page 11, paragraph 1: Kappa value for IHC scoring from different pathologists.

  This was an excellent question. Kappa can provide more information than a simple calculation of the raw proportion of agreement and is the most commonly reported in the medical literature. We asked to our independent statistician to calculate the kappa statistic (or kappa coefficient) and the result was 0.78. Now this information is available in the manuscript.

- Page 11, paragraph 4, sentence 1: replace “was” with “were”.

  Done

- Page 14, paragraph 3, sentence 2: replace “prognosis” with “prognoses”.

  Done

Reviewer # 2 (Dr Etienne Leygue)

Reviewer's report
Version: 1 Date: 18 May 2013

Major comments

- The authors did not mention the existence of variant forms of ER beta that are however clearly expressed in Breast cancer; mainly ER beta 2 or Cx (see: Estrogen Receptor # (ER#) Level but Not Its ER#cx Variant Helps to Predict Tamoxifen Resistance in Breast Cancer, Majida Esslimani-Sahla et al. Clin Cancer
The identification of five major variants of ER-β (β1, β2/cx, β3, β4 and β5), mainly generated through alternative splicing events, increases the complexity of interpreting the literature data accumulated using only one antibody for immunodetection of ER-β expression (Ref). There is no consensus regarding the function of each variant and contradictory results concerning potential function have been published (Ref). It seems that the variant ER-β isoforms can modify both ER-α and ER-β1 activity when co-expressed. Therefore, differential expression of the ER-β variants may play a role in the so-called bi-faceted ER-β action and sensitivity to antiestrogens during breast tumorigenesis and breast cancer progression (Ref). Our immunostainings were carried out using a monoclonal anti-ER-β antibody (clone 14C8 from GeneTex), which is pan-specific for ER-β isoforms. Therefore, we evaluated total ER-β protein levels by performing immunohistochemistry using this well-characterized antibody, previously shown to be one of the best-performing antibodies for this application (Ref)

The references used in the manuscript are:


- Figures should be added to present detailed example of staining for each antibodies, to illustrate cases considered positive and negative for ER beta and alpha, and show examples of ratio<1, between 1 and 1.5 and >1.5.

  As previously requested, we have added a figure (new Figure 2) showing immunohistochemical staining pattern in BC samples. We included examples of ER-β (liver as negative control, whole section positive control slide and Allred scores), ER-α and Ki67 expressions.

  In addition we have added a figure (new Figure 4) to illustrate examples of ratio<1, between 1 and 1.5 and >1.5.

- Legend of Figure 3 is far too incomplete and this figure is potentially not called in the result section.

  The reviewer is absolutely correct. The use of this figure doesn’t increase the clarity of the article. Two of the reviewers of our manuscript requested more information about this figure.

  The initial idea was to investigate whether a correlation (parallel or reciprocal relationship) between ER-α and ER-β existed in treatment groups. The graphs of this figure show the expression level of each receptor and were constructed only to calculate the Spearman’s correlation coefficient (exploratory endpoint).

  The titles or legends of this figure are incomplete and do not include new information to make the figure self explanatory and the results of this specific statistical analysis are written in the main manuscript (Result section). So, we decided to be conservative in using visual elements excessively as their use may cause unwanted confusion and this figure was removed of the manuscript.

- The authors said they calculated the "ratio" ER alpha/ER beta: what was then done when ER beta score was zero?
We added this information and the text now appears as follows:

“If the denominator (ER-β score) of the fraction was zero, we considered as ratio > 1.5 (patients with a much higher ER-α than ER-β score). The exception was when the numerator (ER-α) was zero too. In this case (ER-α = zero and ER-β = zero), we considered as ratio < 1”.

- Many important details are missing: how many cases belong to the ratios classes defined, how many exactly are negative for ER alpha in the different categories etc... Additional Table could solve this issue.

To answer these important questions, we included a table (new table 2) indicating how many cases belong to each study group and the number of ER-α-positive in the different categories.

Minor issues

- Recent review on ER beta should be included. The author could mention and discuss Yan Y. Ann Oncol. 2013 Apr 11. [Epub ahead of print]


  We added the study data in the Discussion as followed:

  “Recently, Yan et al (ref) analyzed ER-β and its co-regulator Steroid Receptor RNA Activator Protein (SRAP) expression in tissue microarrays from a randomized, placebo-controlled trial and found that the benefit was only in the tamoxifen-treated but not in the placebo arm; therefore providing evidence that ER-β expression was predictive for response to tamoxifen inhibition of tumor growth and survival particularly in ER-α-negative premenopausal early BC”.

- In Table 2, it seems that Ki67 is lower in ER beta negative used in placebo (mean and median) than in anastrozole and tamoxifen? Could that not affect the variation? This should be addressed.

   It would even be possible. However, as this actually happened with the placebo group, we do not consider that this fact compromises the data analysis of the other groups (anastrozole and tamoxifen). Some studies suggest that the change in the percentage of Ki67-positive tumor cells after treatment was more strongly associated with recurrence-free survival than the absolute percentage of Ki67-positive tumor cells before treatment.

- In this table, the sum of anastrozole post treatment is 24 patients... should not it be 25 according to table 1?

   Well spotted. The reviewer studied the manuscript. Thanks again for the considerations of our manuscript!

   Table 2 shows the changes after treatment of Ki67 biomarker in ER-β-negative and ER-β-positive cases. Some tumor samples obtained at the time of diagnosis and/or during definitive surgery had insufficient invasive cancer in the biopsy. As one of the cases of anastrozole group had insufficient material at time of the first biopsy (pre-treatment), it was excluded of this analysis (Ki67 variation).

   As the Table 1 data show only the mean pre- and post-treatment Allred scores for ER-β, this case (insufficient material at time of the first biopsy) was not excluded. That’s why there are 25 patients in post-treatment of the anastrozole group in Table 1.

- Page 18, change of ratio alpha/beta during breast tumorigenesis was first shown by Leygue et al 1998, Cancer research 58, 3197 not ref 12.

   The reviewer is absolutely correct. This information was added in the Discussion as well as the reference revised accordingly: *Altered Estrogen Receptor α and β Messenger RNA Expression during Human Breast Tumorigenesis*. Etienne Leygue, Helmut Dotzlaw, Peter H. Watson, and Leigh C. Murphy. Cancer Res 1998;58:3197-3201.
Reviewer # 3 (Dr Sadako Akashi-Tanaka)

Reviewer's report
Version: 1 Date: 19 May 2013

This is a study to see the effect of ER-β and ER-α and their expression change in a short neoadjuvant endocrine treatment setting, which is small pilot study but is of interest. I have following comments.

- In the abstract, one of the purpose of this study is to see whether different regimens have any effect on ER-β and ER-α expression levels. But in the conclusions this was not clearly answered. In order to answer this, statistical analysis should be conducted between the different treatment groups. (Major Compulsory Revisions)

  Well spotted. The reviewer is absolutely correct. We evaluated only the ER-β scores over time among the groups (anastrozole, tamoxifen and placebo) with an ANOVA with repeated measures using rank transformation. The sentence in the Abstract (objective) now appears as: “whether different regimens have any effect on ER-β expression levels”. This specific finding is described in the Results as: “The frequency of ER-β expression did not change after treatment (p = 0.33)”.

- Does the first sentence of the 3rd paragraph in the result section means that “not a significant variation among different treatment” or “significant change of Ki67 levels during neoadjuvant treatment”? The latter one seems to the correct. In the abstract "variation" seems also to be incorrect (Minor Essential Revisions)

  The text has been changed and now appears as suggested: “significant change of Ki67 levels”. We also substituted the word “variation” by “change” in the Abstract and in the sixth paragraph of the Result section.

- Figure 3 tells us the change of expression of ER-β and ER-α during neoadjuvant endocrine therapy stratified by different regimens. But the title of this figure and the
sentence in the result section is positive correlation between ER-# and ER-#. Figures should be drawn some other way. (Major Compulsory Revisions)

The reviewer is absolutely correct. The use of this figure doesn’t increase the clarity of the article. Two of the reviewers of our manuscript requested more information about this figure.

The initial idea was to investigate whether a correlation (parallel or reciprocal relationship) between ER-α and ER-β existed in treatment groups. The graphs of this figure show the expression level of each receptor and were constructed only to calculate the Spearman’s correlation coefficient (exploratory endpoint).

The titles or legends of this figure are incomplete and do not include new information to make the figure self-explanatory and the results of this specific statistical analysis are written in the main manuscript (Result section). So, we decided to be conservative in using visual elements excessively as their use may cause unwanted confusion and this figure was removed of the manuscript.

- The authors described that ER-# and ER-# could be better biomarkers than ER-# and PgR in the last sentence in the Discussion section. If you could evaluate the progesterone expression levels in the same specimens and see the Ki67 change, you will have the supporting evidence. – (Discretionary Revisions)

This is really an exciting idea. We’re already starting another study with a larger number of ER-α-negative patients to compare short-term neoadjuvant treatment in ER-β-negative versus ER-β-positive as well as compare Progesterone Receptor versus ER-β.

Minor point

- P17 L14 predictor of clinical outcome is better to say prognostic factor not to confuse “predictive” and “prognostic”.

   The reviewer is correct and we have clarified this sentence. The text has been changed and now appears as follows:
“Promising findings in ER-β-positive/ER-α-negative BC cases have demonstrated that ER-β status is a significant prognostic factor in univariate and multivariate analysis.”