Author's response to reviews

Title: Heat shock protein90 in lobular neoplasia of the breast

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Version: 3 Date: 19 May 2008

Author's response to reviews: see over
Dear Editor,

I am sending for publication to your distinguished Journal the original revised manuscript entitled: “Heat shock protein90 in lobular neoplasia of the breast”.

We have adopted or we have answered to all the revisions indicated by your reviewers except for the suggestion of your distinguished first Reviewer to change the title into the synonym lobular intraductal neoplasia.

We adopted the most recent WHO classification “lobular neoplasia”. This term does not refer to lobular carcinogenesis as a whole but to a specific entity (precursor lesion; atypical lobular hyperplasia (ALH) and lobular carcinoma in situ (LCIS) according to the most recent WHO classification (Introduction paragraph 3). However, if you insist we will change the term from lobular neoplasia to lobular intraductal neoplasia; I am looking forward to your decision.

In the next page please find attached the point-to-point reply.

Yours sincerely,

George C. Zografos, 
Assoc. Professor of Surgery 
University of Athens

List of changes

Reviewer 1: Harriet Kluger 

Major revisions:

“The idea of looking at HSP90 expression specifically in lobular lesions is excellent. However, the manuscript could be significantly strengthened if it were to include a LARGE cohort of invasive lobular carcinomas 

LCIS is widely considered a hallmark of cancer rather than a pre-malignant condition. Therefore, without the inclusion of invasive lobular carcinomas, there are no prognostic or therapeutic implications to this study, and the last paragraph of the introduction is misleading. 

Please change the title to read ”Lobular intraductal neoplasia“ to avoid confusion, unless invasive lesions are included.”
We would like to thank you for the encouraging comment regarding the idea of the study. Unfortunately, the inclusion of invasive lobular carcinomas was not possible, because such material was not available. Additionally, a statement in the limitations of the study, concerning the issue of invasive lobular carcinomas, is present (discussions section, paragraph 4).

As a result, in the last paragraph of the Introduction, the statement referring to prognostic or therapeutic implications has been eliminated.

We used the term LN (lobular neoplasia) as it is referred to WHO consensus 2002 and we did not adopt the synonym LIN. According to WHO classification lobular neoplasia does not refer to lobular carcinogenesis as a whole but to a specific entity (precursor lesion; LCIS and ALH). However, if you insist on your comment we could adopt the term that you proposed.

“The differences in HSP90 expression between adjacent normal tissue and LN are small. The data might be more impressive if graphically presented (rather than as a description), e.g. using box plots.”

- In the revised version of the manuscript, the two statistically significant findings pertaining to Hsp90 expression (intensity and Allred score) are presented in a graph. The appropriate statement has been added in the text (Results, paragraph 3, last line).

“Not being a statistician, I would like to know what the difference is between the Wilcoxon test and t-tests. Are they the same?”

- Wilcoxon tests are non-parametric tests equivalent to t-tests, but are more appropriate when deviation from the normal distribution occurs. Indeed, that is the case of the present study; scores are not normally distributed, and thus non-parametric (Wilcoxon and Spearman) tests have been performed. A respective statement has been added in the Materials and Methods section (Materials and Methods, last paragraph).

“If additional cases can be added, this might increase the statistical significance of the findings.”

- Unfortunately, as mentioned above, no additional cases were available; as a result, the statistical significance levels were not modified.

At any case, however, the observed findings pertaining to hsp90, both at the level of intensity score and to Allred score, were statistically significant. This may point to the fact that the sample size, relatively limited though, was adequate for the demonstration of significant findings (p-value is the direct expression of type I statistical error).

“Minor revisions: Adjacent normal breast tissue is not actually normal in this case - please discuss in the discussion section.”

- In Figure 1 strong cytoplasmic HSP90 immunoreactivity in the epithelial cells of lobule with slight dilation of the acini is shown. A sentence has been added in Figure legend 1.
Reviewer 2: David Ross Sibson

“(Major revision 1)
The main focus of this manuscript appears to be the expression of heat shock protein (Hsp) in association with estrogen receptors alpha and beta in Lobular Neoplasia (LN) of the breast. The introduction provides a useful background to the role of Hsps, Hsp90 in particular and the significance of the latter for advanced cancer, prognosis and as a therapeutic target. This serves as justification to determine Hsp90 levels in LN as the results could have implications for the relative risk associated with these borderline lesions and suggest opportunities for chemo-prevention. This could have been brought out more fully and I therefore suggest a major compulsory revision to set out more fully the context of the study.

- The Introduction has been extensively revised so as to present the justification to determine Hsp90 levels in LN more fully; indeed, three paragraphs have been substantially enriched (paragraphs 2, 4, 5) in that direction. In addition, the implication for the relative risk together with the opportunities of chemoprevention has been incorporated into the Introduction (last paragraph, lines 2-5).

“In conjunction with similar efforts with regard to the results and discussion this will provide an opportunity to improve the impact of the findings which currently are presented in a relatively neutral fashion. In presenting Hsp90 for example more mechanistic details are available and could be included.”

-Mechanistic details putatively underlying the present study have been added both at the Introduction section (paragraph 4) and at the Discussion section (paragraph 2).

“Reference 6 is emphatic that Hsp90 levels are increased in cancer yet the introduction simply states that it’s expression is abnormal.”
-In the revised version, the study by Pick et al is appropriately discussed. The increase in Hsp90 levels is clearly stated, together with its association with poor prognosis.

“Being definitive with a fuller description would allow the significance of the low levels of Hsp90 seen in the LN as reported in the results to be better presented and appreciated.”
- The Discussion has been extensively revised, adopting a more definitive approach; encompassing molecular mechanisms and critically commenting on the results (cf. Discussion, paragraphs 1-3)

“(Major revision 2)
The spectrum of atypical lesions found in breast is and likely will continue be the subject of much review, discussion and debate. The second sentence in the third paragraph of the introduction is therefore questionable and the reference 13 alone is not adequate or a direct reference. The consensus WHO classification of 2002 and references therein for example would be better or at least included as a (first) addition.”
- The previous reference 13 has been replaced by a new reference (Schreer I, Luttges J: Precursor lesions of invasive breast cancer. Eur J Radiol 2005, 54:62–71) summarizing the recent WHO classification. The definition of lobular neoplasia has been slightly rephrased in the revised version, following word-to-word the aforementioned article.
“Similarly, a more detailed introduction to the estrogen receptor positivity in the lesions of interest would be beneficial, especially given that they are part of the subject matter for the study. Although the majority are positive for estrogen receptor alpha for example this is not necessarily exclusively the case and comments to this effect would be desirable.”

-In the revised version of the manuscript, detailed information regarding ER expression in LN is provided; indeed, a wholly new paragraph has been added in the Introduction (paragraph 5).

“(Major revision 3)
Finally, Hsp90 interacts with a variety of proteins so more should be made of why the hormone receptors in particular have been singled out. Perhaps for example with some discussion of the receptors themselves and their positivity / or negativity as appropriate. What are they considered to do? This would help again in establishing the significance of the findings reported in the results section.

-In the revised version, the fact that Hsp90 interacts with a variety of proteins with particular relevance to breast cancer is discussed (Introduction, paragraph 4). The rationale behind the choice of ERs in the present study is also presented in the following paragraph (Introduction, paragraph 5), together with previous results of our team on ER expression in LN.

“Additional sample, example references that may be helpful are provided below:
P. Workman, F. Burrows, L. Neckers, And N. Rosen
Drugging the Cancer Chaperone HSP90: Combinatorial Therapeutic Exploitation of Oncogene Addiction and Tumor Stress
Silencing of HSP90 Chaperone AHA1 Expression Decreases Client Protein Activation and Increases Cellular Sensitivity to the HSP90 Inhibitor 17-Allylamino-17-Demethoxygeldanamycin
Joanna L. Holmes, Swee Y. Sharp, Steve Hobbs, and Paul Workman
Cancer Res 2008 68: 1188-1197.”

-All references have been incorporated in the Introduction together with brief relevant statements. As a result the second paragraph of the Introduction has been totally revised.

“The methods are appropriate. A key and valuable feature of the materials is the use of LN from presumed cancer free individuals. This has limited the number of cases on which the conclusions are based.”

-We would like to thank you for the comment adding to the validity of the results presented in the study. No changes have been as a result of this comment.
“(Minor essential revision 1). Antibodies for ER beta have been controversial, a note of justification for choice would be helpful”

- We made some trials to standardize the dilution of the antibodies and we concluded that we had satisfactory results with the one that we used, i.e. ER6f-11 and ER-beta (clone EMRO2) Novocastra supplied by Menarini.

(Major revision 4)

“Based on the information I received and there was nothing in the manuscript to suggest I should expect more it is impossible to comment on the results. Only 2 immuno-histochemistry images are provided and these concern the detection of Hsp90.”

- In order to render the manuscript interpretable changes have been made both at the level of results presentation (ER data) and of figures (ER figures).

“It is appreciated that the ER data may have been reported elsewhere but it should be included here to provide context. Only statistical measures are provided and there is no data tabled to allow either the statistics to be checked or the overall nature of the data to be evaluated. It is impossible to know for example how much heterogeneity was observed for example in association of ER alpha or beta with Hsp90.

What are the proportion of ER alpha negative/ ER beta negatives, alpha positives / beta negatives, alpha negatives / beta positives, alpha / beta positives and to what extent were the alternatives associated with the Hsp90 measures”

- In the revised version, the data regarding ER expression have been added to ensure global presentation of the phenomenon. To define ER-alpha and ER-beta positivity/negativity, the criteria presented elsewhere have been adopted (Mann GB et al, J Clin Oncol 2005, 23:5148-5154 and Harvey et al, J Clin Oncol 1999, 17:1474-1481). (Materials and Methods, paragraph 4)

Importantly, all LN cases were positive for both ER-alpha and ER-beta, as the observed percentage of positivity was 10%-90% in all LN cases. This is clearly stated within the revised version (Results, paragraph 4). As a result, only one profile (alpha positive / beta positive) was observed, not allowing the examination of putative associations implicating alpha/beta alternatives with Hsp90 expression.

In the same paragraph, data about ER expression have been added to provide context, as the reviewer requested.

ER beta can be difficult to detect. Has any attempt been made to subclassify the lesions and if so what sized groups are obtained and are there any new associations that are possible as a result? A major revision is required to give a fuller description of the results.

- There was no attempt to subclassify the lesions as they are included in the same term according to the WHO classification.

“(Minor essential revision 2)

Note the current data limitations above. Accepting the statistical significance it is left to rationalise the reported low levels of Hsp90. This would be simply explained if the LN studied are benign lesions containing cells not yet subject to
the stresses occurring in invasive cancer and thus a stress response has not been triggered. That the levels are lower than even normal breast could be a systematic variable brought about by the nature of the cells characteristic of the lesions. Given that classifications have not been reported in the manuscript more is needed.”

- The role of the stress response in the interpretation of the study findings has been integrated in the Discussion of the revised version (Discussion, paragraph 1, lines 8-10); indeed, this comment seems in line with the notion of self-limiting processes in benign lesions, already present in the original version.

“(Minor essential revision 3)
Para 2 discussion. Whilst Hsp90 is essential to the function of ER alpha and beta it is not obvious that these proteins alone should actually be associated with Hsp90. More discussion and justification is needed in this regard. Technical limitations in terms of possible lack of reproducibility are acknowledged in the manuscript.”

- According to this remark, a comment on the expected association between ER and Hsp90 levels has been added; evidence coming from in vitro studies have been presented (Discussion, paragraph 2).

Comments concerning the ease of detection of the estrogen receptor beta in particular would be welcomed. (Minor essential revision 1 above).

- No essential difficulty has been faced in the detection of the estrogen receptors beta. Additionally, ER-immunostaining has been observed on some lymphocytes. A sentence has been added (Results section, paragraph 1).

In the main the authors adequately refer to their earlier work and that of others. See exceptions above. A fuller description of their earlier work regarding the estrogen receptors in their target lesions should be considered.

- According to this comment, the earlier work of our team on ER expression in LN is described in a more detailed manner (Introduction, paragraph 5)

“(Discretionary revision 1)
The title is limited but probably appropriate.”

- The title has not been changed.
Reviewer: Leonardo Della Salda

Reviewers report:
“The authors describe the use of immunohistochemistry in studying heat shock protein 90 and ERs alpha and beta in human breast lobular neoplasia. Results are statistically analyzed for significant association in this possible precancerous stage.

The paper is concise and the significance of the immunohistochemical findings is commented on briefly, in line with the scarce literature available on lobular neoplasia. The results of the study and their explanation are in my opinion, argued correctly in the discussion. The manuscript makes use of the few key references available, with a comprehensive and up to date bibliography.”

-We would like to thank you for the encouraging comment. No changes have been made following this general comment.

“Opinion.
My opinion is that the subject is likely to be of interest to pathologists and the manuscript warrants publication. However, it is not acceptable in its present form.

Major Compulsory Revisions

Abstract
The objectives of the study need to be defined more clearly

-According to this comment, the background of the abstract has been totally revised. Previously published evidence about Hsp90 in breast cancer is provided, whereas the importance of the Hsp90-ER association is clearly stated as an aim of the study.

“Introduction
The manuscript makes use of key references to neoplasia but should focus on breast neoplasia, and not other kinds of tumours.”

- The previously included references, together with the relative statements pertaining to bladder cancer and ovarian cancer have been eliminated. (Introduction, paragraph 2).

Materials and Methods

Immunohistochemistry

“1. The paper does state if or how negative controls were assessed. Please indicate.”

Negative controls were assessed by omitting the primary antibody. A sentence has been added according to the Reviewer (Materials and Methods section, paragraph 2)

Results

“1. Did the expression of the different markers studied differ in an atypical lobular hyperplasia with respect to a lobular carcinoma in situ?”

- The laboratory of our affiliation has adopted the most recent WHO classification (Introduction paragraph 3) and thus provides the term “lobular neoplasia” for the diagnosis and designation of the examined lesions. No data on the specification atypical lobular hyperplasia of lobular carcinoma in situ existed, as a result of the strict adherence to the WHO classification.

“Minor Essential Revisions

1) Results: the authors should insert arrows indicating the nuclear staining in Fig. 2 as they may not be obvious to non pathologist readers.”
- Arrows have been added in Fig. 2 according to the Reviewer.

2) Discussion: A paper is not cited correctly (number 7); this paper examined immunohistochemical HSP90 status in different carcinomas in situ. To date in veterinary pathology, no differentiation has been observed between ductal or lobular in situ carcinoma and there are no differences from a prognostic point of view. In fact this type of carcinoma is only known as carcinoma in situ in veterinary pathology.

- To avoid any confusion, the incorrectly cited reference by Romanucci et al has been eliminated. As the reviewer noticed, the analogies between veterinary and human pathology may be misleading for the audience.

Discretionary Revisions

“I) the report should include images of ER expression. ER staining is not shown. A low-power field figure should be provided.”

- ER-alpha and ER-beta images in LN have been added.

“2) the manuscript interprets the results on the basis of published literature. However, I believe the discussion would benefit from a wider bibliographical analysis of papers on breast malignant neoplasia, where markers have been examined in mastopathical or benign mammary lesions or where the nuclear localization of HSP has been reported (e.g. Yano et al. 1996, Jpn J Cancer Res 87:908-15)”

- According to the comment, wider bibliographical analysis has been incorporated both in the Introduction and the Discussion sections (cf. paragraphs 1,2,4 of the latter). As a total, 11 new references have been added and discussed throughout the text.

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

- English has been revised where appropriate. The changes are highlighted within the text.
Editor’s comments

“Ethics
Experimental research that is reported in the manuscript must have been performed with the approval of an appropriate ethics committee. Research carried out on humans must be in compliance with the Helsinki Declaration (http://www.wma.net/e/policy/b3.htm), and any experimental research on animals must follow internationally recognized guidelines. A statement to this effect must appear in the Methods section of the manuscript, including the name of the body which gave approval, with a reference number where appropriate.”
- The respective statement has been added at the end of the Materials and Methods’ section.

“Informed consent must also be documented. Manuscripts may be rejected if the editorial office considers that the research has not been carried out within an ethical framework, e.g. if the severity of the experimental procedure is not justified by the value of the knowledge gained.”
- The respective statement has been added at the end of the Materials and Methods’ section.

“Manuscript format
Please also ensure that your revised manuscript conforms to the journal style (http://www.biomedcentral.com/info/ifora/medicine_journals). It is important that your files are correctly formatted.”
- Formatting changes have been made throughout the manuscript. Format errors present in the references of the previous version have been corrected.

We strongly encourage you to include an Acknowledgements section between the Authors’ contributions section and Reference list. Please acknowledge anyone who contributed towards the study by making substantial contributions to conception, design, acquisition of data, or analysis and interpretation of data, or who was involved in drafting the manuscript or revising it critically for important intellectual content, but who does not meet the criteria for authorship. Please also include their source(s) of funding. Please also acknowledge anyone who contributed materials essential for the study. Authors should obtain permission to acknowledge from all those mentioned in the Acknowledgements. Please list the source(s) of funding for the study, for each author, and for the manuscript preparation in the acknowledgements section. Authors must describe the role of the funding body, if any, in study design; in the collection, analysis, and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication.
- An acknowledgment section has been added. Additionally, the funding source has been indicated in the Acknowledgment section.