Author's response to reviews

Title: Upregulation of Claudin-4, CAIX and GLUT-1 in distant breast cancer metastases

Authors:

Laura S Jiwa (l.s.jiwa@umcutrecht.nl)
Paul J van Diest (p.j.vandiest@umcutrecht.nl)
Laurien Hoefnagel (l.hoefnagel@me.com)
Jelle Wesseling (j.wesseling@nki.nl)
Pieter Wesseling (p.wesseling@vumc.nl)
Cathy B Moelans (cmoelans@umcutrecht.nl)

Version: 4
Date: 26 September 2014

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

First of all we would like to thank you for reviewing the manuscript “Upregulation of Claudin-4, CAIX and GLUT-1 in distant breast cancer metastases” by Laura S Jiwa et al for publication in BMC Cancer. Herewith we would like to reply to the reviewer’s comments and advised revisions.

Reviewer 1

Major Compulsory Revisions:
1. When stating the aims of the work at the end of the Introduction, the authors state that they “evaluated how this would impact molecular imaging and targeted therapy”. However, to make this evaluation, the author would have to present results showing the use of these proteins for both ends. Please remove the sentence or rephrase, replacing “evaluated” by “hypothesised” or similar.
Additionally, if the authors decide to maintain the sentence, the Discussion must be improved in accordance, as this impact in molecular imaging and targeted therapy is only slightly discussed.
We replaced ‘evaluated’ in the introduction by ‘hypothesised’ accordingly.

2. Figure 1 must also include immunohistochemical results for EGFR and IGF1R.
We have added these results to Figure 1.

3. Authors should provide tables showing the frequency differences that explain the significant p values obtain when evaluating the clinicopathological significance of the negative to positive changes.
The frequency differences have been added in the manuscript text (Results section). It was found to be more informative to add those values in the text itself instead of providing just a table.

4. Besides analysing the clinicopathological significance of negative to positive changes, authors should also provide associations between the clinicopathological features and the expression in primary tumors and metastasis, individually and discuss those results, in light of the results from the literature.
The associations between clinicopathological features and expression in primary tumors and metastasis individually have been analysed and added to the manuscript: there was a significant association between expression of CAIX and GLUT1 in the primary tumor and high MAI.

5. In Discussion, authors try to explain the association of CAIX with adjuvant therapy. Were samples collected before or after treatment? Did the authors take this important aspect in account when performing the statistical analysis?
Unfortunately, we do not know whether patients received adjuvant or neoadjuvant therapy as materials were anonymised some time before this study. We are currently setting up a new database and are trying to complement the information. For this manuscript, we changed all words regarding this topic to ‘(neo)adjuvant’.

6. The authors found an association of CAIX upregulation with younger patients and GLUT-1 upregulation with older patients. Besides the fact that the authors ignored the former in the Discussion, how do they explain that CAIX and
GLUT-1, which are discussed simultaneously in result of their common features (hypoxia-induced proteins involved in the Warburg effect), show opposite associations?

We thank the reviewer for picking this up, which we should have ourselves. For this query, we set double checked the database and labelling of the samples. Unfortunately, we discovered a mistake in the reading of the SPSS output. Correct re-interpretation resulted in some alterations of the results, specifically with reference to the associations of the clinicopathological results and the biomarkers. There was no longer a significant associations between Claudin-4 and lymph node status, and between CAIX and GLUT-1 and age. On the other hand, we did find a positive association between Claudin-4 and tumor size and between GLUT-1 and (neo)adjuvant therapy and time to metastases. We deeply apologize for this mistake for which there is no excuse. Nonetheless, we think that we have rectified this fault correctly and thereby taken away the concerns of the reviewer with regard to the previously reported faulty correlations.

Minor Essential Revisions:
1. In the Abstract, the frequencies of expression change (positive to negative and negative to positive) for each protein are not the same as shown in the Results section. Also, if (n.s) is used in negative to positive change, it should also be used in positive to negative change.

   We apologize for this inconsistency. The frequencies of expression have been altered in the Abstract, in order to match those in the Results. In addition, ‘n.s.’ has been added to the’ positive to negative change’ as well.

2. In the Introduction, the authors state that differences in protein expression when comparing primary tumors with their metastasis is “a process generally known as receptor conversion”. However, the only references provided are from the group and a quick search in the literature showed no other study referring to this phenomenon as “receptor conversion”. In fact, by reading only the Abstract, it is not clear what is “receptor conversion”, and the first relation made by someone who is not familiarized with this expression is with protein conformation, localization, degradation, but not level of expression. Please adjust the manuscript in accordance.

   We have explained the phenomenon of receptor conversion in the purpose of the abstract and the introduction and removed the word “generally”. The following sentence was added: ‘..several studies have shown that the immunophenotype of distant breast cancer metastases may differ significantly from that of the primary tumor, especially with regard to differences in the level of hormone and human epidermal growth factor receptor 2 (HER2) protein expression, a process known as receptor conversion’.

3. In the Material and Methods, the authors state that they selected 97 pairs from a group of 254. Which were the criteria for inclusion/exclusion? Also, why CAIX only present results for 52 cases?

   We have now explained the criteria on which the samples were selected. The selection was based on the presence of sufficient material for TMA construction, as many metastases are small biopsies not eligible for TMA. Also, we explained in the Materials and Methods section that cases with cytoplasmic staining in either the primary tumor or paired metastasis were left out of the analysis. That is why we presented CAIX results of only 52 cases.

4. Why metastatic carcinomas in the bone were marked with H&E if they were excluded from the study?
We apologize for the confusion. The phrasing regarding metastatic carcinomas in the bone has been removed from the manuscript.

5. Please include the negative and positive controls used in the immunohistochemical procedure.
   These had been included and were now described in the manuscript: the positive controls comprised normal breast tissue for Claudin-4, tissue harvested from mice injected with human tumor cells expressing CAIX for CAIX, placenta tissue for GLUT-1 and breast cancer tissue for EGFR and IGF1R. Negative controls were obtained by omission of the primary antibodies.

6. How was the semiquantitatively scoring performed? How was it used in the statistical analysis if cases showing plasma membrane in one out of three cores were considered as positive? Please include this information in the Material and Methods.
   We have now explained in the Materials and Methods section that the scoring of the stainings was performed by regarding a core positive when there was any membrane staining and that a sample was considered positive if at least one out of three cores was scored positive. We omitted the word ‘semiquantitatively’ from the manuscript for clarity.

7. Figure 1 lacks scale bars and magnification.
   Scale bars have been added to Figure 1 and the magnification is mentioned in the legend.

8. Reference 28 does not show that GLUT-1 indirectly regulates intracellular pH. Rather, it shows that GLUT-1 is expressed in the same tumors that express MCT1, an important pH regulator. Please adjust the Discussion in accordance with the reference.
   The sentence and corresponding reference have been replaced by a more appropriate phrasing and reference (see reference 20).

9. In the Discussion, when referring to HIF-1#, please replace “which is maintained by IGF1R” by “which can be maintained by IGF1R”, as IGF1R is not the only HIF-1# inducer.
   Thank you for this suggestion. We modified the sentence as suggested.

Discretionary Revisions:
1. In the Discussion, the authors refer that the upregulation of Claudin-4 in breast cancer metastases occurs especially in lymph node positive cases; however, this result is not provided in the Results. If a result is referred in the Discussion, it should be shown in the Results.
   This has been left out of the discussion, as there was no longer a significant association between claudin-4 and lymph node status after re-interpretation. See point 6 of the major revisions for more elaborate clarification.

2. In the Discussion, the authors refer the preanalytical an analytical variability that should be taken into account. However, they do not further discuss this matter. Can the authors elaborate more on this aspect?
   We now address ‘preanalytical and analytical variability’ in the discussion, explaining that preanalytical variability comprises fixation and processing of the tissue, and that analytical variability involves the staining and scoring of IHC.
Minor issues not for publication:
1. In the end of the first paragraph of the Abstract, there is an extra space after the text.
   We have removed this extra space from the abstract.
2. In the Abstract, “upregulation” and “negative to positive conversion” are used to transmit the same idea. Please refer equally to avoid reader’s confusion.
   We have changed “upregulation” to ‘negative to positive conversion’ for uniformity.
3. In the last paragraph of the Introduction, a parenthesis is opened but not closed.
   This has been adjusted in the manuscript.
4. In the Results, second paragraph, Claudin-4 results for positive to negative changes are inverted when compared to the other proteins (percentage in parentheses and absolute numbers outside parenthesis).
   This has been altered accordingly.
5. The statistical analysis performed was not of correlation but of association.
   Please replace “correlation” by “association”.
   We have substituted ‘correlation’ for ‘association’.
6. Please adjust the last sentence of the Results as it is not clear.
   We have adjusted this sentence as follows: ‘the number of available samples at different metastatic locations were too small for statistical analysis’.
7. A reference is lacking from the first paragraph of the Discussion (Such conversion was previously described for ER, PR and HER2 receptors).
   We have added a suitable reference (see reference 5).

Reviewer 2

Minor Essential Revisions:
1. Upregulated and downregulated cases for each protein or merely the negative to positive changes. Based on Table 1 these values appear to represent both changes, but this may be stated more clearly in the abstract. In addition, it would have been more informative to perform a comparative case-matched analysis of the entire expression sum at each site using the Wilcoxon Signed Ranks test and provide this value for each protein.
   Our apologies for the confusion. We have now addressed this comment in the abstract by the following statement: ‘From primary breast cancers to their distant metastases there was positive to negative conversion, e.g. protein expression in the primary tumor with no expression in its paired metastasis,’. As we have scored the IHC staining as an on/off phenomenon (see comment 3), a Wilcoxon Signed Ranks test is not appropriate. To avoid any further confusion, we have replaced “upregulation” by “negative to positive conversion” throughout the manuscript.

2. Abstract, Conclusion: The authors may consider deleting the word ‘probably’ in the final sentence.
   We have now deleted “probably” in that sentence, and replaced it by “thereby”.

3. Materials and methods: What was the IHC score cut-off? Was any expression considered positive?

See reviewer 1, comment 6 of minor essential revisions. We have explained in the manuscript that the scoring of the stainings was performed by denominating a core positive when there was any membrane staining and that a sample was considered positive if at least one out of three cores was labelled positive. We removed the phrasing ‘semiquantitatively’ from the manuscript.

4. Reference # 20 appears to be in non-English language and should therefore be removed from the manuscript.

This reference and related sentence have been altered and have been replaced by a more appropriated phrasing and reference (see reference 29).

5. Figure 1: Staining examples for all 5 antibodies should be shown.

The Figure has been adjusted to depict all 5 proteins.

We sincerely hope you are satisfied with the rectifications and adjustments in the manuscript. If there are any questions, we are more than willing to respond and clarify any of the statements made above.

Yours sincerely,

Cathy B Moelans, PhD
Dept of Pathology (H04.312)
University Medical Center Utrecht
Heidelberglaan 100
PO Box 85500
3508GA Utrecht
The Netherlands
Tel: +31 88 7556882
Fax: +31 88 7569593
E-mail: cmoelans@umcutrecht.nl