Author's response to reviews

Title: ESR1 Gene Promoter Region Methylation in free circulating DNA and its Correlation with Estrogen Receptor Protein Expression in Tumor Tissue in Breast Cancer Patients

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Author's response to reviews: see over
Authors’ response to reviewers’ comments

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Response to Reviewer # 1 (Dr. Heidelinde Fiegl)

MAJOR COMPULSORY REVISION:

Comment 1: The authors have addressed the major part of the reviewers comments. But the novelties of the findings are only marginal. Therefore the authors should explain in more detail the novelty and the advancement of their findings.

Response: Done. We have expanded somewhat on the value of our findings, whether marginal or not (page 14; lines 11 to page 15 line 7).

Comment 2: Muller et al. could not identify an association between the detection of free circulating methylated ESR1 promoter DNA in serum samples and the oestrogen receptor expression in tumour tissues (Cancer Res. 2003 Nov 15;63(22):7641-5). This controversial result should be discussed.
Response: We thank the Reviewer for highlighting these findings in this article which we had not included in the original manuscript (it has been included in the revised m/s). It indicates the importance of methylation as an independent prognostic factor in patients with breast cancer. Among the results published in Cancer Research are the findings that methylated “RASSF1A and/or APC serum DNA was strongly associated with poor outcome, with a relative risk for death of 5.7” and, as such, the authors concluded that APC and RASSF1A methylated is an important prognostic factor in breast cancer. However, we had not stated that the article described results in relation to absence of correlation between methylation in peripheral blood of ESR1 and the absence of expression of ER in tumor tissue. In our study, we quantitatively analyzed the level of methylation of ESR1 DNA in peripheral blood and its correlation with the non-expression of ER determined by immunohistochemistry in tumor tissue. We found that the level of ESR1 methylation in peripheral blood was >0.02 relative units [20, 21] and was correlated with absence of expression of ER in tumor tissue. According to these results we concluded that methylation can be responsible and can explain, at least in part, the silencing of the ER expression in patients with breast cancer, with the important consequence of explaining resistance to hormonal treatment. Results along this line have been published by Widschwendter M. et al. [29]. The novelty of our study was not only the correlation of ESR1 methylation with ER silencing in tumor tissue, but also the distinct tumor phenotypes of breast cancer that are currently observed in the clinic and which constitute an important prognostic factor. As such, the molecular subtypes of breast cancer with poor prognosis (Triple Negative and Her2) have quantitatively measurable higher levels of peripheral blood ESR1 DNA methylation and, in turn, lower expression of ER in tumor tissue than those subtypes with better prognoses (Luminal A and Luminal B).

Comment 3: The authors describe that “authors such as Widschwendter et al. [28] described a relationship between the APC methylation status and cases of ER+ breast cancer.” But this statement is not correct. Widschwendter et al. described a non-statistically significant association (due to a correction for multiple comparisons; p=0.03) between the ESR1 DNA methylation and the ESR1 expression in breast tissues. They describe that the ESR1 gene, encoding oestrogen receptor, proved to be the best predictor of progesterone receptor status, whereas methylation of the PGR gene, encoding progesterone receptor, was the best predictor of oestrogen receptor status. But they don’t discuss a relationship between APC DNA methylation and the oestrogen receptor. So the statement “In our literature search, we did not find any studies that correlated the epigenetic profile of methylation and its relationship with ER expression status and luminal phenotype” is not correct. Both ESR1 DNA methylation of tumour tissues (e.g. Widschwendter et al.) and ESR1 DNA methylation of free circulating DNA (e.g. Muller et al.) have been correlated to the oestrogen receptor status in the tumour.

Response: We are sorry that this paragraph appears to have caused confusion. We have tried to rectify this aspect in the revised m/s. It now reads:

“In our literature search, we did find a few studies that correlated the epigenetic profile of methylation and its relationship with ER expression status, but we...
found no study correlating the methylation with luminal phenotype. There have been earlier studies that investigated the role of methylation of various genes in search of independent markers of prognosis in breast cancer [28]. However, methylation of the promoter region of the \textit{ESR1} gene has received little attention in relation to the absence of ER expression in the corresponding tumor. Authors such as Widschwendter \textit{et al.} [29] described a significant relationship (p=0.015) between the \textit{APC} methylation status and hormonal receptor status as predictors in patients with breast cancer (revised m/s page 12, line 19 to page 13 line 3).

Comment 4: The legend to figure 1 must be more detailed and the title must be changed since in the presented version it is a little bit confusing. Also the term “\textit{ESR1} test” must be revised in “\textit{ESR1}-DNA methylation”.

Response: We apologize, again for lack of clarity. We have re-written the legend to avoid confusion. It now reads:

“Histogram showing breast cancer subtype of poorer prognosis (TN and Her2) have higher percentage of \textit{ESR1}-DNA promoter methylation > 0.02 relative units, while those phenotypes with better prognosis (luminal A and luminal B) the percentage of \textit{ESR1}-DNA promoter methylation is < 0.02 relative units”
Reviewer # 2 (Dr. Luca Magnani)

MAJOR COMPULSORY REVISIONS:

Comment 1:  Reviewing manuscripts without a proper rebuttal letter really complicate the life of reviewers, at least in my opinion. It was very hard to see which kind of changes were done to the manuscript.

Response:  The opinion of the Reviewer is, indeed, valid. It is of deep concern to us that the Reviewer did not receive our itemized response/rebuttal cover letter. Clearly, it is not in our best interest, and that of our manuscript, to intentionally increase the tedium and to complicate the life of the Reviewer. For completeness, we are re-attaching the original itemized response (see below: “Additional material”)

Comment 2:  What is the status of $ESR1$ promoter methylation in the primary? Can you use the same PCR method used in the fcDNA? Correlation with ERα status is not sufficient. Interest would be elevated if the authors demonstrated correlation between DNA methylation status between primary and fcDNA

Response:  This is an interesting point worthy of further research. However, our study did not have the objective of analyzing the methylation status in tumor tissue. The objective of our work was to quantify the $ESR1$ methylation in peripheral blood and, subsequently, to assess the relationship with ER silencing in tumor tissue excised from patients following surgery. The essential motive for our study was to evaluate methylation in samples that are readily available in an outpatient clinic, such as a simple blood sample. It is increasingly important in oncology to achieve better diagnostic and prognostic data that are most easily obtained without patient distress. Our results do conform to this objective in that, from a simple peripheral blood sample, we can propose an approximate prognosis and therapy.

Comment 3:  What about tumour heterogeneity? What was the % of ERα positive cells in the primary? Could it be that fcDNA comes from the ER- compartment of a ER+ tumour?

Response:  Clearly, the tumor is heterogeneous and, in the same tumor, there can be different clones of cells some expressing ER and others not expressing ER. However, when the measurement of ERα is performed with immunohistochemistry using DAKO system, the overall intensity of tissue section staining is based on the parameters established by expert pathologists as intense staining (+++), moderate staining (+++) and low staining (+). Also, the nuclear uptake of the stain is evaluated as the percentage stained nuclei of the overall tissue section. As such, when it is established that the tumor expresses ER it is because the majority of the cells express ER. Similarly, when quantitatively analyzing the level of methylation of peripheral blood $ESR1$ DNA, starting from the blood taken from the patients, we analyze the overall level of methylation of $ESR1$ in peripheral blood. From the analyses, a level of methylation of $ESR1 >0.02$ relative units we consider a pathological level of methylation. According to our previously published results, levels of $ESR1$
methylation >0.02 were significantly related to the presence of cancer. Conversely, the levels of ESR1 methylation <0.02 were related to the absence of breast cancer. Finally, when we perform the correlation analyses between methylation of ESR1 DNA in peripheral blood and non-expression of ER in the tumor tissue, we note that the result is statistically significant. Nevertheless this relation was not between levels methylation of ESR1 DNA> 0.02 and ER expression.

Comment 4-5: The final conclusion “The results are promising in terms of early diagnosis, monitoring of response to treatment as well as a prognostic factor predictive of response” Page 14 is a gross overstatement not supported by any data. The author now has changed it to” The results are promising in terms of breast cancer prognosis as well as a factor predictive of response to hormonal treatment”. This reviewer fail to see the difference.

Response: We apologize for being too categorical in our conclusion. We have softened the statement in the revised text to read: “The methylation of ESR1 in peripheral blood correlates significantly with the non-expression of ER in excised tumor tissue. As such, this measurement may add prognostic value in identifying luminal phenotypes with poor prognosis and, as well, those with potentially greater resistance to hormonal treatment” (page 15, lines 12 to 15).

“This could be of considerable interest because such an easily measured analyte (ESR1 DNA methylation in peripheral blood) can serve as biomarker, and probable therapeutic target against breast cancer” (page 15, lines 24 to page 16 line 2)

Additional material
Original criticisms of the manuscript and our itemized response-to-reviewers

Reviewer # 2 (Dr. Luca Magnani)

MAJOR COMPULSORY REVISIONS:

Point 1: The manuscript is poorly written. The authors should reformat the manuscript, streamline and simply [sic] the prose and communicate the, [sic] results in an ambiguous [sic] way.

Response: We have sought the help of a native-English-speaking professional provider of editorial assistance to revise the complete manuscript with a view to improving clarity of presentation (scientific and orthographic) while eliminating any errors of grammar, style and syntax.

Point 2: The authors fail to mention until the very end of the discussion the rationale of their design. PBC cells do not express ERAlpha hence the promoter should be methylated. I assume their results imply that difference in ESR1 promoter


methylation observed in pbc is accounted for by difference in the nature of tumor circulating cells. However no reference is provided and there are serious concern about the results. Indeed, the authors report that only 28% and 36% of Luminal A and Luminal B patients have pbc with \textit{ESR1} methylated. This number seems too low considering the limited amount of tumour \textit{sic} circulating cells in the plasma. Could the authors describe in better details how the \textit{sic} prepare the pbc cells? Do they enrich for tumour \textit{sic} circulating cells?

**Response:** Perhaps we were nor clear in our methodology statements. The study established the \textit{ESR1} methylation status using free circulating DNA (fcDNA) in plasma of patients with breast cancer. We related the findings to the ER expression in tumor biopsy tissue using immunohistochemical techniques. We did not isolate peripheral blood cells (pbc) nor circulating tumor cells. In the revised m/s we have clarified these concepts wherever they had arisen.

**Point 3:** It is not clear what is the real advancement in the field. The author states in page 12 that “In our literature search, we did not find any studies that correlated the epigenetic profile of methylation and its relationship with ER expression status”. This statement leads this reviewer to think that the authors perform they \textit{sic} search quite superficially. Here \textit{sic} few examples of previous work that looked specifically at the DNA methylation profile of ESR1 promoter in breast cancer tissues: Maybe the author stated that there are not \textit{sic} studies that linked pbc \textit{ESR1} methylation and E\textalpha status? What is the benefit of their approach compared to normal histo-pathological staining?

**Response:** We thank the reviewer for highlighting the dangers of claiming priority for one’s findings without having conducted as exhaustive a literature search as would seem desirable. What is new in our study is that it was conducted in Caucasian patients, and that we analyzed the methylation of \textit{ESR1} as a factor involved in the silencing of ER receptor, not only triple negative but Her2, as well. Further, in comparing the subgroups with better prognosis (i.e. luminal A and B) we found that the methylation was significantly lower than the subgroups of poor prognosis. Further, the results point towards the possibility of each phenotype of patients with breast cancer can have subgroups with a greater or lesser level of methylation of \textit{ESR1} that could have an effect on overall survival with treatment. These findings were significant despite a low number of patient samples. Finally, our results highlight a possible future field of research such as the use of de-methylating agents as therapeutic tools which may reverse the expression of ER- to ER+ and, as such, not only generate a new hormone therapy but also, perhaps, improve the prognosis of these patients by converting them to ER+ expression.

**Point 4:** The statement “Hence, it appears that the molecular variant of the promoter of the \textit{ESR1} gene…” Page 13 is not supported by any results. Table 5 is missing.

**Response:** Perhaps we had overstated our claims, and these have been omitted in the revised m/s. The table 5 had been deleted in an earlier version of the manuscript. Sorry for the error.
Point 5: The final conclusion “The results are promising in terms of early diagnosis, monitoring of response to treatment as well as a prognostic factor predictive of response” Page 14 is a gross overstatement not supported by any data.

Response: As with Point 4 (above), we may have been a trifle over-optimistic. We have toned down these statements in the revised text and have confined ourselves merely to a low-key interpretation of our findings.