Author's response to reviews

Title: MicroRNA profile in very young women with Breast Cancer

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Author's response to reviews: see over
Dear Sir/Madam,

Thank you for giving us the opportunity to submit a revised version of our manuscript. We would also like to thank the reviewers for their positive feedback and constructive comments and suggestions made. We believe we respond to all comments posed by the reviewers below. Likewise, the manuscript has been revised accordingly and changes made are highlighted in yellow.

Reviewer's report
Title: MicroRNA profile in very young women with Breast Cancer
Version: 1 Date: 2 February 2014
Reviewer: Patricia Casbas-Hernanez

Reviewer's report:
The manuscript ‘MicroRNA profile in very young women with Breast Cancer’ seeks to distinguish breast cancer that arises in very young patients (less than 35 years old) from those that arise in older patients (older than 65 years old) using microRNA profiling. It is a well written document with a clearly stated question; it combines the emerging field of microRNA with an important clinical dilemma. The findings may have implications for how breast cancers in young women are managed. However there are certain aspects of the methods, analysis and limitations of the study that should be reconsidered:

- Major Compulsory Revisions

1- In the description of the study population it would be relevant to know if the biopsies used for the study were from primary breast cancer or if it was a recurrence. Also, if women that participated in this study had a family history of breast cancer and their BMI.

We thank the reviewer to point us that there is not a clear specification about the tumor used in the study being primary or recurrence from a previous disease. Yes, indeed every biopsy used in this study was obtained from primary breast cancer and obviously before any treatment (this last statement was used in the original work). Approximately a 25% of the young samples used in the study had known family history of breast cancer recorded, however none of them has mutations in BRCA1/2. All patients with such family history have been submitted to a counseling unit in order to perform a BRCA test according to the guidelines in St. Gallen International Expert Consensus.
In order to get this very clear in the corrected manuscript we have modified the corresponding text in the section materials and methods. We have also included BMI information as suggested by the reviewer as a new clinical characteristic in Table 1.

2- The majority of papers the authors reference are studying ethnically diverse populations. African American women are known to have higher rates of cancer at young age (and more aggressive). The authors should state the ethnic makeup of their population, how their population is different/similar to the previously published populations, as well as explain how
their study is more/less appropriate to answer their question and what limitations or strengths does their population present when trying to extrapolate their findings to other populations.

We agree with the reviewer that this information was not obvious from the text. The reason is that the great majority of patients ascribed to the hospital are of Caucasian origin. We had now defined properly the ethnic origin of patients enrolled in the study in material and methods section. And so, we have in our sample set all Caucasian women of Spanish or European origin, except for one sample that has a indigenous of South American ascendance, which in fact displays a different pattern compared with the remaining young samples, resembling more to the older women included in the study, we have commented all this in the text. Our data may not imply similar molecular alterations in women from African American descent; however, it is representative of a Caucasian population and for this, our conclusions could be extrapolated and relevant for the scientific community. Some comments have been added to the discussion regarding this point.

3- The authors did not provide any information regarding their 3 ‘normal’ patients. It is important to know the characteristics of these patients (age, BMI, etc).

Yes, we agree this data may be important, however, the normal breast tissue samples available were obtained from mammary reduction surgery performed on healthy young women, of whom we only known the age and we don’t have access to any additional clinical information. The use of these samples allows us to discard miRNA signature intrinsic of young women. We thought this was clearly explained in the main text, however, we have added more concise information related to age in the corrected version.

4- There are no methods for explaining how histologic staining was made or how markers were measured. Please provide description of how the subtypes were defined (Luminal A, Luminal B…).

Thanks for the appropriate remark, we have now added this information into materials and methods section specifying in more detailed the histological procedures considered.

5- The authors state that all tissue samples contained more than 30% of tumor material; the variation from 30% to 100% is a big range of epithelial content and may explain some of the differences observed among young and old tumors. This is a variable that should be included in table 1 and adjusted for in the analysis. Breast density decreases 1-2% every year in normal circumstances; this change in breast density implies a change in the epithelial and stromal contents of the breast. Therefore, young breasts have more epithelium than older breast which have more stromal content. These changes in breast composition are accompanied by changes in gene expression patterns when whole tissue gene expression studies are performed (Relationship of mammographic density and gene expression: analysis of normal breast tissue surrounding breast cancer. Sun et al. Clin Cancer Res.2013 Sep 15;19(18):4972-82.). Hence, microRNAs expression in whole tissue may also be influenced by the amount of stroma and epithelium within a tumor.

All tumoral material was selected by two expert pathologists in breast tissue whom authored the paper. All procedures were made with standard protocols based according to pathologists’ recommendations. Moreover, the fact that none of the deregulated microRNAs in tumors from young women matches the expression of the three healthy samples discards the hypothesis that the differences observed in miRNA expression were due to age-related issues independently of breast cancer (as changes on mammary density are).
6- When assessing potential confounders for the microRNA profiles in the discovery set, the authors study the influence of tumor size, nodal status, ki67% and histological grade. However, they should also study the influence of tumor subtype in this discovery data set (the same way they adjust later in the manuscript for the validation test set).

We did not correct the discovery set for subtypes because we have already selected a set of samples balanced between young and older women according to their subtype, ensuring us that both sample sets are representative of all subtypes.

- Minor Essential Revisions
7- Figure 3 is missing axis labels, please add labels for classification.
8- Dates and timelines for tissue acquisition and RNA processing are important elements to add to the methods.

We have corrected mentioned minor issues.

Reviewer’s report
Title: MicroRNA profile in very young women with Breast Cancer
Version: 1 Date: 3 February 2014
Reviewer: Jodie Fleming
Reviewer’s report:
Major Compulsory Revisions:
1. The authors provide a general comparative evaluation of miRNAs differentially regulated in tumors from young women and elderly, post-menopausal women. This limited study describes a set of miRNA that were differentially regulated in women of various ages, and while the concept could be informative to those intimately related in the field, the authors fail to perform any functional studies to confirm the function of the miRNA highlighted and their possible role in breast cancer cell behavior. Other studies have shown miRNA directly regulating cell motility, chemo-sensitivity, methylation of stem-related genes, etc. Similar functional studies should be included.

We agree that functional studies and further analyses would enrich the study but we consider that the identification of a distinctive miRNA profile is relevant enough to be shared with the scientific community. However, the objective of our study was to determinate whether a miRNA profile could be related with breast cancer in young women.

2. Generally, qRT-PCR analysis is not sufficient for validation of such a broad conclusion in such a widely-read and diverse audience BMC journal.

Although the reviewer points to the inaccuracy of the qRT-PCR to validate the profile identified, it has been demonstrated to be an effective method (Rosenwald 2010) and it has been used for validation in previous recent works of similar characteristics published in BMC group journals (Kang2013, Qi2012).

3. Moreover, a significant number of important patient demographics were not accounted for in the analyses that should be addressed to gain more insight from the current data presented within the manuscript.

Age, gender, geographic area, and now BMI and ethnic origin, cancer subtype and diagnosis are included together with many other prognostic factors. We are not fully aware of the
important demographics that are not accounted for as the reviewer has mentioned, because most of the considered demographics contemplated in the early breast cancer consensus of St. Gallen is present in our data.

2. As the authors are comparing young women with significantly higher levels of circulating estrogens to the older, post-menopausal women, an analysis of the newly identified miRNAs and how they may relate to estrogen signaling is a must. It is currently unknown if the tumors evaluated in this study are simply documenting miRNA regulated by estrogens. If the authors were to perform such an analysis, this may be of interest. Perhaps reporter assays or other in vitro analyses? If the authors were to test a set of miRNA for response or regulation by estrogen the studies would be a welcome contribution. Indeed the authors highlight some of the previous studies that have linked miRNA to estrogen signaling (refs 27/28), but do not follow up on this note.

We sincerely thank the advice made by the reviewer and agree that the study of the implication of circulating estrogens would make a contribution. In order to minimize this contribution we used breast tissue from healthy young female. Our profile does appear different from these normal samples and so, the analyses do not point out directly to oestrogen related miRNAs. Indeed, some miRNAs modulate estrogen signaling, however, none of the most significant ones in the study fall in this category. In addition, none of the pathways obtained in the enrichment analyses of the selected clusters were associated with estrogen metabolism directly.

3. In addition to circulating hormone levels between pre- and post-menopausal women, the issue of parity must be related to the authors’ findings. Pregnancy, lactation and involution are known to significantly influence and even reprogram breast tissue. It is suggested that the age and number of pregnancies can affect breast cancer onset as well as influence the differentiation state or stem cell population. The current study does not account for the parity status of the women from which the tumors were obtained. The status of the number of children and age at first birth would shed some light into the difference observed in miRNA expression. Was the alteration in miRNA levels simply due to pregnancy?

25% of the young patients had already had children at a very young age but do not correlate with any molecular genetics deregulation detected with the microRNA study. And so, we can exclude pregnancy as the reason behind alteration in miRNA profiles. We have now included this data and comments on the main text.

However it should be considered that there are important inherent differences between younger and older patients. In general it can be said that young patients have more estrogen exposure, parity is more recent, do more physical activity, have different diet, smoke more and have less pharmacologic exposure to treatments like antihypertensive than older ones. All this exclusive epidemiological differences are considered inherent in both groups.

We cannot reject an influence of these factors in the tumor profile, but the objective of this study was not to identify which of the epidemiological factors cause a difference between older and younger women induce breast cancer. Our objective was to identify whether there is a profile that describes breast cancer in young patients or not, and we are indeed presenting such profile.

Unfortunately, our study was not designed to clarify whether these differences are due to a specific epidemiological reason. For instance we only can speculate if an increased rated in the
parity of the older patients or if a reduced but more recent parity in the younger ones may be the reason for such findings.

4. The correlation between patient BMI and miRNA should be considered. As the current literature is continually highlighting the importance and influence of BMI, obesity and metabolic syndrome on breast cancer risk and behavior, it would be most helpful to know the BMI status of the patients analyzed. The possibility exists that the miRNAs identified are differently regulate due to metabolic status.

We appreciate the suggestion of the reviewer. According to the suggestion made, we have obtained the BMI data of the patients. The average 22.34 BMI of BCVY is normal and the older are overweight (29.74 Kg/m²). However, this risk factor acts differently in young women, being lower BMIs risk, while in older women higher BMIs result in elevated risk of developing breast cancer. This is the reason why BMI is treated as a factor with qualitative age interactions in many breast cancer studies (Anderson2009, van der Brandt2009, Kawai M, 2014). We consider that the factor cannot be tested in both populations (young women and old women) merged since the relation of BMI with breast cancer is not comparable in both groups. We add this information in Table 1.

5. Used ki67, size, nodal status, histological grade but not intrinsic molecular subtypes. A more in-depth analysis of the molecular subtype may have shed some light into the observed differences in cell motility/invasion, adherens junctions and cell adhesion molecules. Indeed, the authors state that the younger women suffer from “more aggressive’ phenotypes and other studies have “revealed miRNA expression to be specific to breast cancer intrinsic subtype” but fail to evaluate or relate any of the current studies observations to molecular subtype. Did the previous studies relating intrinsic subtype account for age? What were the demographics in comparison to the current study? The authors need to express how their study enhances the current knowledge in the field and currently is not convincing as a comprehensive analysis comparing the limitations and results of the numerous other studies is lacking. If the intent was to strengthen the argument presented on page 13 that breast cancer in young women is “a distinct entity beyond the intrinsic breast cancer subtype” then more functional and in depth analyses are needed.

As we have answered to first reviewer, we countered the possible confounding effects given by subtypes by selecting balance sets of patients according to their subtypes. In Figure 2 the distribution of the subtypes among groups of samples can be seen, demonstrating no aggregation of clusters according to their subtypes. We want to emphasize the fact that the clustering method grouped the samples by their age and not by subtype for the 121 miRNAs with significantly different expression in young women. We are sure that there are many other miRNAs that could separate the samples by their subtype, but in our study we focused on miRNAs that had a different expression patterns according to age.

6. A very similar study was recently published, though the authors argue that their results “pointed to genes barely implicated in the pathways of found in our study”. It would be helpful for the authors to discuss their results in more depth in comparison to the highly similar study conducted by Colak and Cols: their current argument is that the inclusion of six samples under the age of 35 was the reason for such a large discrepancy in results between the two studies.

We are thankful for this recommendation, we have realized the lack of discussion with this data, and we have made changes accordingly, as well as commented more studies previously published when considered relevant. However, the study of Colak and cols was performed on
messenger RNA data and not from microRNAs. Moreover, the study has only 5 samples with 35 years old or younger patients, and 24 samples with ages between 35 and 45 years old. And for clustering analyses all women below 45 years old have been pulled together, and so the 83% of the samples in this category are over our class of young patient.

Almost none of the genes detected coincide with our data with the exception of integrins, WNT related genes that explain the more invasive phenotypes in young samples. We hope that the discussion is clearer now as well as is the aim of our study.

**Minor Compulsory Revisions:**

*Shift in font size throughout*

We have now corrected this.

We appreciate the reviewers’ comments, thanks to which we have revised thoroughly the discussion section and reorganized it appropriately.

We believe we have been able to explain all reviewers’ comments, doubts and suggestions. However, we will be happy to provide further information if required.