Reviewer's report

**Title:** Importance of pre-analytical steps for transcriptome and RT-qPCR analyses in the context of the phase II randomised multicentre trial REMAGUS02 of neoadjuvant chemotherapy in breast cancer patients

**Version:** 1  **Date:** 31 October 2010

**Reviewer:** Jorge Reis Filho

**Reviewer's report:**

Major reviews

- This study is NOT MIAME guidelines compliant and the microarray-based gene expression data were NOT deposited in a public repository. At this day and age, this is utterly unacceptable and needs to be rectified prior to any resubmission.

- The microarray analysis methods are described in such a sketchy fashion that this reviewer would be unable to reproduce the analyses described in this manuscript. Following the results of Ioannidis et al. (Nat Genet 2009), who were unable to reproduce the results of several high profile studies on gene expression profiling owing to lack of analysis details, the authors have the onus to provide sufficiently detailed analysis methods. This would be best achieved by providing a supplementary Sweave document (or any form of auto-executable file), similar to those described by Baggerly and Coombes.

- According to the authors, “Tumour cellularity was evaluated on frozen sections of the biopsies dedicated to RNA extraction by local pathologists identifying epithelial tumour cell vs stromal cells, inflammatory cells and necrosis”. This assessment was of crucial impact on the sample selection, given that only samples with >30% of tumour cells were “kept for further analysis”. Despite the importance of this step, the methodology for the assessment of tumour cellularity is subjective. In fact, it is unclear whether the local pathologists assessed percentage of nuclei, percentage of section area or percentage of cytoplasmic area. This needs to be clarified. Furthermore, the method employed by the pathologists to determine these percentages needs to be fully described.

- In the RNA extraction protocols, it is unclear if a step to eliminate completely any trace of ‘contaminant’ DNA was introduced. If that was not the case, how would the authors exclude that some some of the SYBR Green results were not derived from amplification of the genomic DNA? Please clarify and provide the required controls.

- The choice of housekeeping genes is questionable. Some of these housekeeping genes have been shown to have a remarkably poor performance when tumour (rather than cell line) material is used (please see Lab Invest. 2005 Jan;85(1):154-9; Mol Diagn. 2004;8(2):107-13; J Mol Med. 2005 Dec;83(12):1014-24). This reviewer would strongly encourage the authors to include at least a few more robust housekeeping genes.
- Another question that is germane here is whether the pathologists were trained a priori. Given the poor inter-observer agreement between pathologists, different pathologists may have excluded cases that had approx 30% of tumour cells differently and this may have led to biases in sample selection.

- The choice of 30% of tumour cells is debatable. The results of Cleator et al. (Breast Cancer Res 2006) and Weigelt et al. (Lancet Oncol 2010) demonstrate clearly that different percentages of non-neoplastic cells in the samples affects the performance of gene classifiers. Before accepting the 30% threshold based on convenience (i.e. the same threshold used in other studies), the authors should instead do a series of spiking experiments to demonstrate what percentage of non-neoplastic cell contamination affects the performance of qRT-PCR and microarrays for classification of samples by gene expression profiling.

- The fact that Centre 4 is a clear outlier needs to be better explored in the discussion. Is this a mere problem of RNA extraction (Trizol vs other methods)? How would this impact on the interpretation of the results of from material obtained from current clinical trials where different extraction methods have been employed. Are there bioinformatic methods to minimise the impact of different extraction methods or is this an insurmountable hurdle? The authors need to expand on this in the discussion.

- This reviewer cannot subscribe to the authors’ conclusion that “Our data showed that strict quality criteria for RNA integrity assessment and well calibrated and standardized RT-qPCR allows multicentre analysis of genes transcripts with high accuracy in the clinical context.” In fact, this manuscript shows that even with strict quality criteria for RNA integrity assessment and well calibrated and standardised RT-qPCR, there was centre bias in the gene expression analyses. Without offering a solution for the centre-based bias, this conclusion is not supported by the authors’ data.

- The high number of cases excluded prior to the analysis owing to low cellularity (~15%) should also be discussed. How can microarrays and qRT-PCR be used if 15% of the samples are not adequate for this type of analysis? It is also important to determine whether there was a difference in terms of tumour size, ER, PR, HER2, grade and histological type between the samples excluded and included in the study. One could hypothesise that the group of samples with <30% of cancer cells is enriched for lobular carcinomas or ER positive tumours.

- Out of the 290 samples with sufficient tumour cell content, 22% and 18% of samples were not available for microarray and qRT-PCR analysis, respectively. Why were the samples excluded? This needs to be explained in greater detail in the methods.

- Out of all samples collected, only 66.4% were successfully analysed by microarrays (i.e. one third of samples were excluded). Again, the impact of this high failure rate needs to be discussed in relation to the design of clinical trials that also address translational research questions based on transcriptomic analysis.

Minor reviews
- The term Elston-Ellis grade is most unorthodox. Chris Elston and Ian Ellis themselves have always referred to their modification of the Scarff-Bloom-Richardson system as “Nottingham Grading System”. Furthermore, the reference Elston & Ellis (Histopathology 1991) should be included in the manuscript.
- The Clinicaltrials.gov reference number and webpage of the trial should be included in the manuscript.
- Some typos were found and need to be corrected prior to resubmission.

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:

I declare that I have no competing interests