Author's response to reviews

Title: Digital imaging in the immunohistochemical evaluation of the proliferation markers Ki67, MCM2 and Geminin, in early breast cancer and their putative prognostic value

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Author's response to reviews: see over
Dear Dr. Solera,

MS: 1702242171555840, Digital imaging in the immunohistochemical evaluation of the proliferation markers Ki67, MCM2 and Geminin, in early breast cancer, and their putative prognostic value; Joshi and Watkins et al.

We would like to thank the Editorial Board and the referees for their contributions to and constructive criticism of our manuscript. We have carefully considered the reviewers’ comments and revised our manuscript accordingly. We believe this now to be a clearer and much improved report into the immunohistochemical scoring of MCM2, Geminin and Ki67 expression via conventional light and digital imaging-based microscopy, as well as the prognostic value of these markers. We would also like to point out that ‘Johnathan Watkins’ is to be considered joint first author.

Below, we have provided point-by-point responses to the reviewers’ comments. Please note that amendments to the manuscript that are in response to the reviewers' comments are highlighted in yellow.

We hope we have clarified the points raised by the referees to your satisfaction and that you now consider the revised manuscript acceptable for publication.

Yours truly,

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Reviewer 1

#1 > “The authors research immunohistochemical evaluation of the proliferation marker Ki67, MCM2 and Geminin in early breast cancer. This paper did not show MCM2 and Ki67 as prognostic marker in multivariate analysis. They cannot derived this conclusion from this data.”

Our response > We appreciate the reviewer’s concern that MCM2, Ki67 and Geminin do not serve as independent markers of breast cancer-specific survival (BCSS) as shown by our multivariable analysis in this cohort of patients. We consider this to be an important finding since it argues in favour of using lymph node status and histological grade as the most important primary prognostic tools in early invasive breast cancer.

However, the cohort of patients we have examined includes a range of tumour subtypes and grades and it is, of course, true that proliferation markers may be of prognostic and predictive value in different intrinsic subtypes and stages of breast cancer, such as hormone receptor-positive and lymph node-negative subgroup. We argue that scoring the expression of such markers via digital imaging technology may prove to be a more efficient means of reproducible prognostication.

We have highlighted these points in the Conclusion.

Reviewer 2

“This is a very interesting topic. Congratulations on your effort in assembling a cohort with long patients follow up data and performing immunohistochemistry for an array of markers.”

Major Compulsory Revisions:

#1 > “In my opinion and according to my experience the cores of 0.6 mm are really small and even if three cores were taken these hardly represent the characteristics of the primary tissues/tumours.”

Our response > We appreciate the point by the reviewer and agree that there is variability in the core caliber used in different series in the literature. However, there are numerous instances in the literature where 0.6mm cores are used in TMA preparations [1-4]. In particular, the benefit of including 3 such cores from different areas of the tumour helps to mediate against tumour heterogeneity, whilst fewer, but larger, cores, runs the risk of not being representative.

#2 > “Were the ER and HER2 immunohistochemistry reperformed on TMA slides or the original data were used? If the data were collected from the original files the same antibodies were applied during the analysed period or different? Please mention the antibodies used for ER and HER2 IHC.”

Our response > The ER and HER2 immunohistochemistry were not re-performed on the TMA slides. The original data from patient information records was collected. For IHC of ER and HER2, we used the SP1 clone (Invitrogen, Leica BOND-Max, 1 in 100 dilution) and Leica Ready to Use kit (Leica, BOND-Max) antibodies, respectively. We have now amended the manuscript’s methodology section and included this information (page 11, line 218-223). We have also listed the antibodies used in Table 1.
The authors have used a mixed bag of cases which includes ER positive, Her2 positive and triple negative cases. First of all the Table nr. 1 it seems interesting. Probably it is not acceptable to consider the cases with not known data as a part of 100%. For example considering the expression of HER2 the authors have found the followings “Positive 26 (8.4%), Negative 129 (41.7%) and Not known 154(49.8%)”. How the authors should know that from the 154 not known cases how many are in reality HER2 positive or HER2 negative. I would strongly suggest to recalculate the data considering the percentages only in the cases with known data. According to this calculation in the case of HER2 IHC approximately 80% of the cases are going to be HER2 negative instead of 41.7%.

Our response > We thank the reviewer for highlighting this issue. We agree and have recalculated the association of proliferation markers with HER2 using the number of cases (155/309) where HER2 report is available and amended Table 4 accordingly.

Have the authors compared for example the Ki67 expression observed on the whole, original tissues with that observed on TMAs. This should be important especially considering the relatively low expression of the Ki67 observed on TMAs. At least in some cases I would suggest to compare the expression of Ki67, MCM2 and Geminin expression of whole tissues with that of TMAs.

Our response > We acknowledge the possibility that IHC expression in whole tissue sections may not concur with that observed on TMAs. However, various researchers studied the concordance between IHC staining on whole tissue sections and on TMA cores and found no significant difference between the two [5, 6]. Nonetheless, we understand that slight discrepancies in expression may well exist and we have stated this as a limitation in the Discussion (page 19, lines 400-406). We acknowledge the fact that every biomarker needs to be validated for its suitability for use in a tissue microarray (page 19, line 395-400). We observed that the inter-core variability of Geminin was significantly high in our study. Consequently, we suggest that tissue microarray methodology may not be suitable for assessment of Geminin because of its generally low expression.

How many of the cases were of LUMA subtypes known to express Ki67 at low level?

Analysing proliferation markers and their prognostic value is difficult given the heterogeneity of breast carcinomas and the different treatment modalities used. As an idea it would be interesting to divide the cases is the known subtypes and analyse if there are significant differences especially in the geminin expression between different subgroups.

Our response > We recognise the insight that would be afforded by an analysis of a luminal A subgroup of cases. Unfortunately, we have not been able to profile the transcriptome of these samples by microarray or sequencing technology. Therefore, we are unable to determine the molecular subtype of each tumour. As a surrogate for the luminal A subtype, which is known to be dominated by hormone receptor-positive,
HER2-negative tumours [7], we examined the prognostic value of these markers within an ER-positive, and either HER2-negative or lymph node-negative (good prognosis) subpopulation. However, the resultant sample sizes (<100) meant that we were underpowered to detect a significant difference in survival between high- and low-expressing cases. Nonetheless, we looked within an ER-positive subpopulation (225 cases) and found the prognostic value of the markers in univariable analyses to be retained (Additional Files 1 and 2). However, in a multivariable analysis, these markers were again not significant (Additional File 2).

In addition, we wish to highlight our examination of the differences in the expression of the three IHC markers between ER-positive and -negative tumours, and HER2-positive and -negative tumours, the results of which can be found in Table 3.

In future investigations, we would like to subtype tumours by their transcriptional profiles and examine the differences in the expression of these IHC markers, and thereby focus on the utility of these proliferation markers more specifically in the Luminal A subgroup.

Minor suggestions:

#7 > “The p values have to be given uniformly and it is not essential to give 5-6 decimals.”

Our response > We have amended our use of p-values so that only 4 decimal places are shown.

Reviewer 3

Despite being of somewhat limited interest this is a well-written and readable paper, which seeks to answer two main questions in a relatively small number of FFPE samples. One question is whether digital microscopy images are as effective as slides viewed down a light microscope for scoring IHC stains. The other question concerns, which of three proliferative markers has got the most value as an additional factor in prognostication.

Suggested discretionary revisions are.

#1 > The starting material is quite old and it is likely that there was an appreciable gap between the original embedding and arraying, this could be clarified (line 211).

Our response > Although the surgical procedures and the FFPE samples are somewhat historic, the TMAs were not made at the time of surgical excision but more recently (in the last 5 years approximately). We, of course, cut new sections for the staining for Ki67, MCM2 and Geminin. This is in concordance with recommendations in the literature in which sections are stained immediately after cutting [8].

#2 > “It appears likely that the same researcher did both the light microscope scoring and the scoring using the digital platform but this is not absolutely clear (line 236).”

Our response > The first author (SJ) scored samples for expression for both methodological approaches but was blinded to the results of the scoring. Approximately 10% of the scores were re-assessed
by SJ, PG and JPB, and there was a general consensus in scoring. We have now clarified this point in the document (page 12, line 236-239).

#3 > “Comparisons of scoring methods have been done on a case by case method but core by core might have been more appropriate (line239).”

**Our response** > As well as case-by-case analysis, we have done a core-by-core analysis to look for inter-core variability (page 15, line 296-301). We found no significant variability for Ki67 and MCM2 but observed a significant core-to-core variation for Geminin. Since Geminin has a low expression, it is perhaps not surprising that a greater number of cells must be counted to evaluate its expression accurately.

Minor essential revisions

#4 > “Line 235, assessment should read assessed.”

**Our response** > We thank the reviewer for highlighting this textual error and have changed ‘assessment’ to ‘assessed’.

#5 > “Line 328, does not flow properly.”

**Our response** > We apologise for the lack of clarity. We have amended the original two sentences:

> Whilst tumour size, lymph node status and HER2 status were not associated with the proportion of tumour cells demonstrating positivity with any of the three proliferation markers, higher histological grade and ER-negative tumours demonstrated higher frequency of expression with all 3 markers (P < 0.001 for all, Mann Whitney test). The details of clinico-pathological correlation are presented in Table 3.

To:

> Whilst tumour size, lymph node status and HER2 status were not associated with these proliferation markers, higher histological grade and ER-negative tumours had higher expressions of all 3 markers (P < 0.001 for all, Mann Whitney test) (Table 4).

We hope this is now clearer.

#6 > “The conclusion section is rather short and although the conclusions appear well supported by the work, it is not clear whether this work adds much knowledge to the field.”

**Our response** > We have amended the conclusion to underline the primary findings and implications of our study. Thus, the following passage:

> Digital microscopy images can be effectively used as a high-throughput technique for assessing the immunohistochemical expression of proliferation markers in early invasive breast cancer
with comparable results to light microscopy-based assessment. MCM2 is a more sensitive marker of proliferation and prognosis than Ki67, and merits further evaluation in future studies on breast cancer proliferation status especially in the good prognosis subgroup such as lymph node negative, hormone receptor positive patients.

We have shown for the first time to our knowledge that digital microscopy images can be used as a high-throughput technique for assessing the immunohistochemical expression of proliferation markers in early invasive breast cancer with results that are comparable to those from light microscopy-based scoring. We used MCM2, Ki67 and Geminin, and found MCM2 to be the most sensitive marker of proliferation and prognosis among the three. Despite not finding these three markers to be independently prognostic of BCSS as evinced by our multivariable analysis, digital microscopy-based assessment of these and others may yet find utility in particular subgroups of breast cancer patients, for example in lymph node-negative, hormone receptor-positive patients, which have generally better prognoses. Future studies using immunohistochemistry should be directed towards utility of Ki67 and MCM2 in choosing the appropriate adjuvant therapy in early breast cancer cases.
References


