Author's response to reviews

Title: Analysis of and prognostic information from disseminated tumour cells in bone marrow in primary breast cancer: a prospective observational study

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Author's response to reviews: see over
Comments to reviewer’s reports on

"Analysis and prognostic information from disseminated tumour cells in bone marrow in primary breast cancer: a prospective observational study" BMC Cancer

A: Reviewer Kasimir-Bauer

1. The authors should only focus on those patients who finally were included in the study (n=401). In this regard, the flow chart can be deleted.

Answer: We agree that focusing on these patients will clarify the manuscript. The flow-chart is now deleted and the text on p 7 modified.

2. Comment on the conclusions drawn in the abstract: It has to be considered that the BM aspirations were performed several years ago with no standard procedure clearly defined.

Answer: We have modified the conclusion sentence in the abstract.

The detection of DTCs in bone marrow in primary breast cancer has been shown previously to be a predictor of poor prognosis. We were not able to confirm these results in a prospective cohort including unselected patients before the standard procedure was established. Future studies with a standardised patient protocol and improved technique for isolating and detecting DTCs may reveal the clinical applications of DTC detection in patients with bone marrow metastasis.

In the introduction, the authors state that BM aspiration is associated with pain and discomfort. In the context of this paper and other studies this is not quite true since all aspirations had been performed during surgery.
Answer:

We have now modified the text in introduction on p 5 reflecting the reviewer’s correct comment and has been excluded in the discussion section:

Aspiration may be associated with pain and discomfort for the patient, particularly if performed repeatedly to monitor treatment.
3. A standard for DTC detection clearly has been defined and has to included in the revised version.

**Answer:** At the time being, there was no standardized technique and therefore we are not able to revise it according to today's standard for all steps. We are sorry for this, but can't write anything that was not actually performed. We hope you find that we in the revised version emphasize that the study was launched before any standard method was published and the findings is now discussed with this in clear mind.

However, we pre-treated the cells in a citrate-buffer in a microwave oven for 20 minutes as a permeabilization method and used a detection method with EnVision™ and NovaRed™ for visualization. We did not use any Mab A45 or a conjugate of Fab-fragment as negative controls and thus we are not able to include this in the method section. We have now included the permeabilization and visualization steps in our method for IC method (which is now a minor part of the publication).

For the IC method, the cells were fixed in buffered formaldehyde (4%) and thereafter **pre-treated in citrate buffer (pH 6) in a microwave oven for 20 minutes.** The cytokeratin antibody kit (AE1/AE3, Daco, Glostrup, Denmark) was used as primary antibodies against CK1,2,3,4,5,6,7,8,10,13,14,15,16 and 19. The EnVision™ (Dako, Glostrup, Denmark) was used as detection system, NovaRed™ (Vector Laboratories, Immunkemi AB, Sollentuna Sweden) for the visualisation and **Mayers hematoxylin for nuclei staining.** This enables direct immunocytochemical evaluation (IC) of the cells and analysis by light microscope (Olympus CX41, Tokyo, Japan) in 74 patients.

4. The positive rate of 25% in normal donors is much too high.

**Answer:** We agree that this is the main draw-back with the study and mirrors the lack of standardized and strict method used at that time. The revised manuscript has now highlighted the importance of standardization and focus
on stressing that the results obtained in our manuscript mirrors these drawbacks at the time of inclusion on p 14-15. This is also highlighted in the general conclusion of the reviewer.

Furthermore we included bone marrow from 76 healthy bone marrow donors and the analyses were positive for epithelial cells in bone marrow in 19 (25%) of the samples illustrating the lack of standardisation of the assays used.

Epithelial positive rates in bone marrow have been reported in non-cancer patients even after morphological criteria was applied (5% and 30% in two different cohorts) [17,26]. The findings in the present study, including 25% positive cases among adult healthy bone marrow donors, raise concerns about the specificity of the method used.

5. The images of DTCs can be deleted.

Answer: We have attached some new images and it is up to the editor to decide if they are to be included.

6. In the discussion the clear focus should be discussion the differences of the methods used.

Answer: We have made substantial changes in the discussion section.

P 12

The present study included patients before the standard protocol was published [15], and the data are mainly derived from detection by an IF staining procedure that was not included in the published meta-analysis and is not advocated by the consortium [7, 15].

P 13

7. Aspects to consider when estimating the results of these early reports are the heterogeneity of the patients included and different methods and techniques used to determine bone marrow dissemination. However, more recent studies performed with standardised methods of detection also propose a prognostic value of DTCs in bone marrow [22-24]. Molloy et al. found clinical significance of DTCs in bone marrow in
terms of BCSS (HR, 2.1; p = 0.003), but not in metastasis-free survival (HR, 1.5; p = 0.127) [23]. Giluiano et al. reported that DTCs were present in 104/3413 (3.0%) patients and was associated with decreased overall survival in univariate analysis, but this did not reach clinical validity in multivariate analysis. They concluded that bone marrow aspiration was not recommended in routine clinical practice for patients with early breast cancer without an improved technique for isolation and detection of occult tumour cells in bone marrow [22]. Solá et al. found a higher frequency of DTCs in a subgroup of patients who experienced breast cancer-related events (13%), but the results did not reach statistical significance because of low power with few events [24].

8. The chapter of CTCs should be excluded

**Answer:** We agree that this is a complex topic and has now excluded this from the discussion section.

**B. Reviewer Georgoulias**

1. The authors describe an immunofluorescence assay for DTCs based on an antibody which can detect a wide range of cytokeratins. Moreover, they use another antibody for the ICH assay. There are several methodological problems which require clarifications before starting evaluate the clinical value of the detected cells in the cohort of patients: what is the the sensitivity and the specificity of the assay?? did the authors performed preliminary experiments using a breast cancer cell line spiked into normal bone marrow cells? did the authors performed experiments using different numbers of bone marrow mononuclear cells in order to define the optimum conditions for the preparation of cytopsins? Did the authors compared the two different antibodies and the two different assays in both CK-positive and CK-negative samples as well as in MCF-7 cells spiked into normal bone marrow cells? This is extremely important if the data should be pooled for the final analysis. The high frequency of CK-positive cells in normal donors raises several questions concerning the specificity of the assay and limits the validity of the prognostication value of DTCs.
Therefore, the authors have to demonstrate that the cells that they characterize as DTCs are really micrometastatic cells.”

**Answer:** We performed experiments with breast cancer cell-lines spiked into normal bone marrow. This was done for both the immunofluorescence assay and the IC assay. These samples were used as positive controls. Before the start of the analyses we also optimized the conditions for cytopsin by using different numbers of bone marrow mononuclear cell suspension. We have not examined the specificity and sensitivity of the assay.

1b. The background has to be appropriately modified.

**Answer:** We have now changed this section according to the reviewers comment on p 5.

Comparisons among different detection methods were performed [17] before standardised guidelines were published [15]. These comparisons showed difficulties in interpreting CK-positive cells as tumour cells and recommended the use of markers that allow discrimination between CK-positive cells of haematopoietic and non-haematopoietic origin.

2. Absence of CK positive DTCs in the Figure 2: according to Fehm et al. 2008, (Breast Cancer Research, 10:R76) a representative DTC of a patient with breast cancer has a high nuclear to cytoplasmic ratio, irregularities in the nucleus and CK stains the cytoplasm at the periphery of the cell causing a ring-like appearance. The authors have to present what they characterize as CK positive DTCs from patients.”
**Answer:** The irregularities in the nucleus and the ring-like appearance were included in our characterization of CK-positive DTCs. This is added in Material and Methods.

The presence of DTCs was defined as cytokeratin-positive cells with disseminated tumour cell morphology (irregular staining of the cytoplasm) with an enlarged nucleus, **irregularity of the nucleus**, a high nuclear-to-cytoplasmic ratio, **CK stains the cytoplasm at the periphery of the cell causing a sing-like appearance** and fluorescence-positive intact cells (immunofluorescence technique) according to Fehm.

3. In the material and methods, the authors reported that they used the breast cancer cell line MCF7 spiked into blood from healthy volunteers as a positive control for CK immunostaining. The authors have to present an image of MCF-7 spiked to normal PBMCs.”

**Answer:** Please find enclosed an image of MCF-7 spiked to normal PBMCs. We think it is for your journal to decide whether this image should be included in the manuscript.

4. The 3 tables are not included.

**Answer:** In our submission they were all included and are included in this submission version again.

5. The results of higher cut-offs are given here but not included in the text.

In 214 of the included patients we had access to enumeration of DTCs using the IF method and has now performed an exploratory analysis of different cut-offs which is not included in the manuscript but will be given here.

The range of DTCs was 0-23 cells and 42% of the patients (n=110) had one or more positive DTC, most of them having only one cell (n=18), two cells (n=11), three cells (n=10) and four cells (n=12). We performed a Cox regression analysis using 5 year DDFS as end-point. We tested number of DTCs as a continuous variable and with a cut-point of 3 and 4 cells in the whole cohort and in the subsets of N0 and N+ patients. The results were similar to the main results in the manuscript showing a tendency towards a
more pronounced effect by DTC in the N0 subset, although no statistical significance was found. The full dataset is given in the table here.

<table>
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<td>≥ 3 cells vs ≤ 2 cells</td>
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<tr>
<td>All patients</td>
<td>1.22</td>
<td>0.55-2.85</td>
<td>0.6</td>
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<tr>
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<td>0.32-16.34</td>
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</tr>
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<td>0.37-2.48</td>
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<td>1.28</td>
<td>0.53-3.10</td>
<td>0.6</td>
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<tr>
<td>All patients</td>
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<td>0.42-21.03</td>
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<td>0.35-2.64</td>
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</table>

Minor revisions

1. P 4 node-negative may be node-positive

**Answer:** We thank the reviewer for this comment and has now corrected this on p 4.

2. AJCC classification

**Answer:** We have now included a comment on this on p 4
There has been an increasing acceptance of DTCs as an independent marker of a poor prognosis in breast cancer and is now included in the new AJCC classification as a diagnostic criteria for micrometastatic spread

3. Correction on p 12

Answer: We have now corrected this sentence on p 12.

The bone marrow from adult healthy donors was analyzed using both methods.

4. Correction on p 13

Answer: We have corrected the grammatics on p 13

The present study included patients before the standard protocol was published [15] and the data are mainly derived from detection by an immunofluorescence (IF) staining procedure.

C. Reviewer Pierga:

1/ From a methodological point of view, it is not acceptable that the results of two different methods, used sequentially and not concomitantly on different samples, can be mixed for the analysis. The vast majority of the patients were screened by immunofluorescence. The results for DFS and OS should be given only for the IF methods. For the ICC method, the number of patients is too small to draw definitive conclusion (74 patients). Even if a subgroup analysis was performed, it
is not appropriate to pool the two methods in the same analysis. This IF procedure was not used in the metaanalysis published by Braun et al in 2005 and is outside recommendations of the consortium published in 2006 by Fehm et al in Cancer 2006. Did the authors compare the detection rate one the same samples with the two techniques (IC and IF)?

**Answer:** We have compared ICH and IF in 21 samples. Fourteen samples (10 negative and 4 positive) showed concordant results, 1 were positive with ICH but negative with IF, whereas 6 showed the opposite pattern (negative with ICH, but positive with IF). The results for both method are given in Table 3, but the KM-plots included in the revised version includes only data from the IF based analysis. We have included a sentence in Discussion p 12:

The present study included patients before the standard protocol was published [15], and the data are mainly derived from detection by an IF staining procedure that was not included in the published meta-analysis and is not advocated by the consortium [7, 15].

2/ More than one hundred patients had bone marrow sampling without any result. This is a very high percentage and is astonishing. What are the reasons for not being able to handle the samples? At least more detailed explanations should be given.

**Answer:** The reason for not analyzing DTC in 168 samples was due to change in research strategy at our laboratory. We also considered that 401 cases should be enough for evaluating the prognostic importance of DTC.

3. comment on healthy donors
Answer: The healthy donors were bone marrow donors and there was a specific informed consent for them. We have changed this on p 12

The bone marrow from adult healthy bone marrow donors was analyzed using both methods.

4. Median-follow up

Answer: The median follow-up time (61 months) has now been included on p 7

5. Sternal crest aspiration

Answer: The spelling is now changed to sternum. The double aspiration from the sternum was performed because we wanted to be sure to have enough bone marrow aspirate for the analysis (parallel to what is used from bilateral iliac crest aspiration).

6. Concerns about the high rate of positive cases.

Answer: We do agree that this is a major concern and reflects the lack of standardized technique at the time when the patients were included. However, this is an interesting observation that might raise concerns about other older publications in the field. We have further addressed this concern in the Discussion section on p 14:

Furthermore we included bone marrow from 76 healthy adults and the analyses were positive for epithelial cells in bone marrow in 19 (25%) of the samples. This illustrates the lack of standardisation of the assays used. As cytokeratin positive samples were found among the healthy donors in the present study, we cannot exclude the possibility that some of the DTC+ breast cancer patients were wrongly classified.

7. Quoting recent studies.

Answer: We have now included the mentioned references as proposed on p 14.
Aspects to consider when estimating the results of these early reports are the heterogeneity of the patients included and different methods and techniques used to determine bone marrow dissemination. However, more recent studies performed with standardised methods of detection also propose a prognostic value of DTCs in bone marrow [22-24]. Molloy et al. found clinical significance of DTCs in bone marrow in terms of BCSS (HR, 2.1; p = 0.003), but not in metastasis-free survival (HR, 1.5; p = 0.127) [23]. Giluiano et al. reported that DTCs were present in 104/3413 (3.0%) patients and was associated with decreased overall survival in univariate analysis, but this did not reach clinical validity in multivariate analysis. They concluded that bone marrow aspiration was not recommended in routine clinical practice for patients with early breast cancer without an improved technique for isolation and detection of occult tumour cells in bone marrow [22]. Solá et al. found a higher frequency of DTCs in a subgroup of patients who experienced breast cancer-related events (13%), but the results did not reach statistical significance because of low power with few events [24].

Minor discussion

1. Reference nr 22

**Answer:** Reference nr 22 is now correctly quoted as seen above

2. Quality of the photos

**Answer:** We have enclosed some X-tra photos for optional use.