Author's response to reviews

Title: St Gallen molecular subtypes in primary breast cancer and matched lymph node metastases - aspects on distribution and prognosis for patients with luminal A tumours. Results from a prospective randomised trial.

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Author's response to reviews: see over
Dear Editor,

Thank you for the review of our manuscript and the opportunity to send a revised version of the manuscript MS: 8194227359620642 “St Gallen molecular subtypes in primary breast cancer and matched lymph node metastases – aspects on distribution and prognosis for patients with luminal A tumours”, which we have prepared according to the reviewer’s comments and authors instructions including a suggestion for a new title “St Gallen molecular subtypes in primary breast cancer and matched lymph node metastases – aspects on distribution and prognosis for patients with luminal A tumours. Results from a prospective, randomised trial”. We have found the comments useful and think the manuscript has improved while incorporating all comments from the reviewers.

Our answers to all comments are given below and the page and line of each revised part is provided. In the manuscript, all changes are highlighted in yellow.

Reviewer 1.

1. The study has been carried out at relatively old material (1985-1994). What was the impact of time on quality of immunohistochemical staining? Were samples from primary tumors and lymph node metastases stained and analysed at the same time? This information should be included in the manuscript.

Answer comment 1:

No immunhistochemical staining was performed at the time of inclusion of the patients. The staining and analysis of all markers were performed retrospectively and simultaneously for evaluation of the primary tumours and lymph node metastases (for ER and PgR in 2002 and for HER2 and Ki67 in 2008). This has been clarified on p 14.

“The immunohistochemical staining of primary tumours and lymph node metastases was performed at the same time”.

2. Why distant disease-free survival was selected as the primary end-point and not overall survival, which up to date remains “gold standard” of survival analysis? It would be valuable to show also correlations of St Gallen molecular subtypes in primary tumors and lymph node metastases with overall survival.

Answer comment 2:
Distant disease-free survival was the primary end-point in the original randomised trial, overall survival the secondary endpoint. However, we have now included data and figures (2b and 3b) on overall survival (Kaplan-Meier plots) in the manuscript showing similar results on p 7 and clarified the end-point on p 16.

“Three degree of freedom log rank tests revealed significant differences in DDFS and OS between the subtypes for both primary tumors (p=0.002 and p<0.001, respectively) and lymph node metastases (p=0.003 and p<0.001, respectively) with the HER2-positive and triple-negative subtype associated with the shortest survival time (Figure 2 and 3).”

“Overall survival was the secondary endpoint and included deaths of any cause”

3. The manuscript presents switch between molecular subtype classification between primary tumors and lymph node metastases. It would be very interesting to see what was the prognostic value of this switch.

Answer comment 3

We agree that an analysis of the prognostic value of this subgroup shift would have been very interesting to study, but due to low power when comparing the prognosis for the 7 shifting patients to that of patients with stable subtypes, we decided not to present this data. We have, however, now added a sentence on page 7 stating that no significant prognostic differences were found between shifting and stable subtypes (data not shown) and commented on the low power in these analyses. For your info, two distant metastases and three deaths were observed in the subgroup of patients with shifting subtype. The logrank P-values when comparing the prognosis in this subgroup to that of stable luminal A (n=38) were (DDFS: p=0.43, OS: p=0.78) and to stable non-luminal A (n=40, DDFS: p=0.32, OS: p=0.18).

“Patients switching from luminal A in the primary tumour to non-luminal A in the lymph node metastases (n=7) had no significant change in prognosis compared to the stable luminal A subgroup (n=38) or to the stable non-luminal A subgroup (n=40) in terms of DDFS or OS (data not shown). However, the number of patients shifting from luminal A to non-luminal A are few, and no definitive conclusions can be drawn from this study”.
4. Besides showing St Gallen molecular subtypes comparison of status of individual biomarker expression (ER, PR, HER2, Ki67) in primary tumors and lymph node metastases should be presented to answer the question if there is really the benefit of combining biological markers rather than assessing them individually.

Answer comment 4

We performed a comparison of individual biomarker expression in a previous study including the present patient cohort, showing high concordance between primary tumor and corresponding lymph node metastasis (Falck et al, 2010, ref # 17). Combining individual biomarkers into surrogate molecular subtypes provides prognostic information with special reference to ER positive tumors, who constitutes about 80% of breast cancer patients (Goldhirsh A et al, 2011, ref #1, Brouckaert O et al, 2012, ref #11), with potential impact on treatment recommendations.

We have rewritten the opening in the discussion section, p 8:

“Combining biological tumour markers into surrogate molecular subtypes has been shown to add prognostic information [6-8, 10, 11] which may be of importance for recommendation of systemic therapy. Unlike the analyses of individual biomarkers in the present cohort of patients [17] which showed high concordance between primary tumours and corresponding lymph node metastases, the molecular subtypes identify a subgroup of patients with ER positive disease as luminal B, with a worse prognosis, who may benefit from adjuvant chemotherapy alongside endocrine treatment [10, 14, 20].”

5. Why HER2 2+ cases were classified as HER2-negative? If no FISH result is available, I would rather suggest excluding those cases from the analysis, as this result is equivocal and some HER2-positive cases may occur within 2+ subgroup

Answer comment 5

We agree with the reviewer that the result of IHC HER2 2+ constitutes a group of tumors with equivocal HER2 status. According to concordance data up to 24% of patients with HER2 2+ are amplified according to FISH analysis (ref. Dybdal, 2005, ref#24). The controversies are further addressed in ASCO/CAP Guidelines which advocates that HER2 2+ tumors should be analysed according FISH in the clinical setting (ASCO/CAP Clinical Notice 2007).
In the present study, 8/85 tumours were classified as HER2 2+. Application of concordance data would yield 1-2 patients with amplification according to FISH and the majority of patients would not be amplified and we decided to include them as HER2-negative. The decision not to exclude these patients was, of course, also affected by the limited size of the cohort.

We have now included a section in Discussion, page 10, last section within the subheading tissue analyses:

“In the present study, 8/85 tumours were classified as HER2 2+ according to IHC analyses. In a national survey performed by our group, 12% of HER2 2+ tumours were amplified according to fluorescence in situ hybridization FISH [24] and in another study [25] the concordance was up to 24%. This would result in 1-2 patients of HER2 2+ tumours as amplified in the present cohort, thus patients with HER2 2+ tumours were included as HER2-negative.”

6. The citation of the paper published by the same group should be added (Falck AK, Bendahl PO, Chebil G, Olsson H, Fernö M, Rydén L. Biomarker expression and St Gallen molecular subtype classification in primary tumours, synchronous lymph node metastases and asynchronous relapses in primary breast cancer patients with 10 years’ follow-up. Breast Cancer Res Treat. 2013 Jul;140(1):93-104) and the authors should comment how the data presented in current manuscript relate to already published one.

Answer comment 6

We have now included a section comparing the two cohorts (ie inclusion criteria, adjuvant treatment, method of HER2 analyses) in the discussion section; page 9, with referral to the recently published article:

“In the recently published study from our group [22] comparison of molecular subtypes in primary tumour and synchronous lymph node metastases also revealed a shift in individual patients. The shift was observed from luminal A to non-luminal A in the metastatic node as well as the reversed shift, from non-luminal A to luminal A in the metastatic node. In the present study, only shifts to a molecular subtype with worse prognosis were
observed. The number of patients in the present study cohort is limited (N=85) and the inclusion was restricted to patients with stadium II breast cancer whom all received adjuvant treatment with tamoxifen irrespective of expression of ER as opposed to the patients in the more recent study [22] which constitutes an unselected cohort where patients were offered adjuvant treatment according to modern guidelines. The analyses of HER2 also differ between the studies, where assessment according to immunohistochemistry (IHC) (present) or silver in situ hybridization (SISH) [22] could affect the results. Interestingly, shifts are observed in individual patients in both patient cohorts according to molecular subtypes, proposing a molecular event in the metastatic niche during tumour cell progression with influence on prognosis.”

Minor Essential Revisions
7. According to St Gallen guidelines besides luminal A, B and triple negative HER2-positive subtype is distinguished so phrase “HER2-type” should be changed to “HER2-positive” subtype at page 5 and following pages

Answer comment 7
We have now rephrased HER2-type to HER2-positive in the manuscript, which is highlighted in yellow.

8. What is the value of putting the titles and legends to figures in additional file? They should rather be placed in the main manuscript.

Answer comment 8
We have now included the legends in the main manuscript.
Reviewer 2:

1. It is unclear how many sections of the same tissue were evaluated for consistency of classification.

Answer comment 1:
Due to the sparse material available, we only prepared one section per tissue and biomarker. This has now been clarified on p 15

“One section per tissue specimen (primary tumour and lymph node metastases) and biomarker was evaluated”.

2. Was at least a sub set of the luminal A subtype that showed a shift evaluated for as a complete section ie. Not at the size of tissue microarray core. It is essential to know if the observations were consistent throughout the section.

Answer comment 2.

The classification of luminal A and non-luminal A was based on tissue microarray staining with two cores. The drawback with this technique has been addressed in the discussion on p 10.

“The method has limitations because a small area of one of the metastatic lymph nodes is examined. Sampling may therefore contribute to bias in representative areas of evaluation”.

3. It will be of interest to state if the disease outcome for these patients worse that others of luminal A.

Answer comment 3:

The question has been addressed under Reviewer 1; comment 3
4. It is also unclear if these patients had any other metastases at other lymph nodes or other sites in their body and how did those sites compare to the primary tumor classification.

Answer question 4:

The inclusion criteria are mentioned on p 13 and states that only patients with early breast cancer stage II were included. For the seven patients with a shift in phenotype, three had one lymph node metastases, two had three metastases, one had six and one had ten lymph node metastases at the time of inclusion. Stage IV was an exclusion criteria, this has now been clarified. We have not collected any distant metastases for biomarker analysis and don’t have access to data regarding location of distant metastases in the patients experiencing distant metastases in this subset of patients.

“with stage II unifocal, radically operated early breast cancer without distant metastases”.

5. Similar information (understandably not statistically significant) will be helpful for the her2 type and triple negative cancers that showed a shift.

Answer comment 5.

We have added a description of these patients on p 7.

“One patient shifting from triple-negative in the primary tumour to HER2-positive subtype in the lymph node metastases had distant metastases and died within one year, whereas the patient shifting from HER2-positive subtype to a luminal B subtype was without any event at 5 years follow-up.”
6. Table 2 and table 3 have figures in parenthesis presumably these are percentages. However that can be clarified in the revised figure legend.

Answer question 6.

We have now added this in the revised figure legends.