Author's response to reviews

Title: Hot flashes are not predictive for serum concentrations of tamoxifen and its metabolites

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Version: 2 Date: 3 October 2013

Author's response to reviews: see over
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Title: Hot flashes are not predictive for serum concentrations of tamoxifen and its metabolites

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Version: 2 Date: 4 October 2013

Author's response to reviews:

Dear editor,

Thank you for providing us the opportunity to improve the manuscript with the comments and suggestions of the reviewers. Please find below in blue the responses to the comments raised in the review. In the revised text, the changes have been highlighted with track changes.

Yours sincerely,

Nynke Jager
Reviewer’s report
Title: Hot flashes are not predictive for serum concentrations of tamoxifen and its metabolites
Version: 1 Date: 15 July 2013
Reviewer: Chi-Chen Hong
Reviewer’s report:

1. Was total time of treatment with tamoxifen considered as either an explanatory variable and/or potential confounder in relationships tested? It’s possible that women treated for a longer period may be more or less likely to experience hot flashes. This should be examined and included as a confounder if relationships between tamoxifen metabolites and time on treatment are related.
We thank the reviewer for this useful comment. We agree that time of treatment might be related to the experienced hot flashes. We added the following sentence to page 3 “The severity of hot flashes is suggested to increase during the first three months of tamoxifen treatment, followed by a plateau or even a decrease for the duration of treatment [Loprinzi et al. (Clinical Breast Cancer, 2000) and Love et al. (Journal of the National Cancer Institute, 1993)].” Also, we included duration of treatment as a potential confounder in the statistical analysis, as is shown in the methods and results section.

2. Table 1 and Table 2 can potentially be combined into one table, with descriptives provided by group (i.e. experience with hot flashes prior to tamoxifen treatment).
We believe that splitting the tables will be more clear and give a better overview, since table 1 contains baseline characteristics and table 2 contains some results of the study. Therefore, we decided not to combine these tables.

3. To better present the data, in addition to correlation coefficients, it may be informative to provide mean metabolite levels (along with SD or 95% confidence intervals if data is skewed) by categories of hot flash frequency and severity of hot flashes as shown below. Frequency of hot flashes can be categorized into 3 or 4 groups, depending on the distribution. These analyses can be further adjusted for potential confounders, including whether a woman was experiencing hot flashes prior to treatment initiation with tamoxifen (yes/no). These analyses can be done in addition to poisson and ordinal regression analysis, which may be more subject to influence of specific data points.
We have provided the additional data as supplementary tables (Supplementary 4).

4. Similar to the point made above, it would be informative to present metabolite levels by CYP2D6 phenotype, with and without adjustments for potential confounders, including experience with hot flashes prior to tamoxifen treatment. Given that CYP2D6 has a substantial proportion of missingness (unknown), it is inappropriate to treat this as an ordinal variable, as shown in table 3. Not clear why only associations with CYP2D6 phenotype are only shown for endoxifen (figure 3). These associations should be shown for all metabolites.
We have provided the additional data as a supplementary table (Supplementary 3). We decided to delete Figure 3 from our manuscript, since this was not the primary goal of our research, and this finding is in line with the literature. We reported the correlations for tamoxifen and its three metabolites in the manuscript (page 8).
5. In the statistical section (page 6), it is stated that missing values were imputed with medians. Proportions of each variable with missing values should be indicated in Table 2. Only for the estradiol concentration analysis and the genotyping some values were missing, this proportion is stated in Table 2. Since this was not clearly stated in the text, we added the sentence: “In all analyses estradiol concentrations were log transformed and missing estradiol and CYP2D6 values due to insufficient material were imputed with population medians.” to page 7.

6. In table 2, it is not clear what “Analyzed patients (n=)” means under “Median estradiol concentration (within detection limits)”. The number of analyzed patients seems very low. If so, how were adjustments made in multivariate analyses? “Analyzed patients (n=)” are the patient samples that resulted in an estradiol concentration above the lower limit of quantitation. Since this was not clear enough, we deleted this part from Table 2 and added the following sentence to page 8 of the manuscript: “For 70 (64%) samples the analyzed estradiol concentration was below the lower limit of quantification (LLOQ, 43 pmol/L).”

7. Figure 2 is not needed given that BMI is really being treated as a confounder in these analyses examining associations between metabolites and hot flash symptoms and is not a primary focus of the paper. Figure 2 was deleted from the manuscript.

8. If figures are provided for relationships with frequency of hot flashes, it is not clear why tables for severity of hot flashes are not provided. In Table 3, both hot flash frequency and severity data are provided, hot flash frequency in Table 3A and severity in Table 3B. In addition to the information shown in these tables, we decided to visually illustrate the association between hot flashes and tamoxifen and metabolite levels. For this purpose, we chose to depict the association between tamoxifen and metabolite levels and hot flash frequency.

9. Results of the sensitivity analyses, with removal of Group II women needs to be discussed in the Discussion section. With adding the information of menopausal status to our cohort, we found pre-treatment flashes to be associated with serum concentrations in postmenopausal women. This is now more extensively discussed in the Discussion section.

Discretionary Revisions
10. In Table 1, in addition to T and N status, would be nice to also include AJCC stage. We have added that additional information to table 1.

Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: Yes, and I have assessed the statistics in my report.
Declaration of competing interests: I declare that I have no competing interests.
Reviewer's report
Title: Hot flashes are not predictive for serum concentrations of tamoxifen and its metabolites
Version: 1 Date: 17 July 2013
Reviewer: Thomas E Mürdter

Reviewer's report:
In a retrospective study, Jager et al. investigated the correlation of vasomotor symptoms with plasma levels of tamoxifen and its metabolites in order to evaluate the usefulness of this common side effect of anti-hormone therapy as a surrogate for tamoxifen plasma levels and outcome.
This is an interesting, well planned study and excellently written manuscript. However, the major drawback of this study is its limited number of patients included. The authors need to address this issue; at least, they should discuss this in more detail.

Major Compulsory Revisions:

Abstract:
Page 2 lines 36f: As there are also reports missing significant correlations between hot flashes and outcome (see discussion) this sentence needs to be modified.
We have modified the sentence to “In addition hot flashes are suggested to be positively associated with tamoxifen treatment outcome.”

Background
Page 3 lines 65: …, is also a potent inhibitor…. In contrast to norendoxifen with an IC50 in the nanomolar range, endoxifen is only a weak aromatase inhibitor with an IC50 of 10µM. Please correct.
We have modified the sentence to: “…unlike 4-hydroxytamoxifen, also inhibits aromatase…”.

Page 3 line 81f: This sentence does not adequately reflect the results of the referenced study. Cuzick et al. used a subset of the ATAC trial to analyze the correlation between treatment induced endocrine symptoms (hot flashes and arthralgia) and treatment outcome in a total of 1997 patients under tamoxifen treatment. Whereas vasomotor symptoms alone were not significantly correlated to survival (HR 0.82 (0.63–1.05), p=0.12) there was a significant correlation between joint pain and survival (0.55 (0.39–0.78) 0.001) which was also present in the subgroup of patients receiving anastrozole. Based on this, one may question why the authors did not include joint pain in their questionnaire. This should be addressed to in the discussion.
We have changed the sentence to “Cuzick et al. investigated whether the occurrence of treatment-related symptoms (vasomotor symptoms or joint symptoms) is associated with breast cancer recurrence. They found a trend that patients using tamoxifen who experienced newly emergent vasomotor symptoms (eg hot flushes, night sweats and cold sweats) had a lower recurrence rate….”
For this study, we were primarily interested whether we could find an association between tamoxifen metabolite concentrations and hot flashes, therefore we did not include joint pain in our questionnaire.
Patients and Methods
Page 4 line 96: Please state that both, pre- and postmenopausal patients were included in the study. This is of importance as estradiol plasma levels are included in the analysis.
The sentence was changed to: “Patients, both pre- and postmenopausal...”. We also included menopausal status into our analyses.

Page 4 lines 98f: As this study is a retrospective study using a rather complex system to evaluate subjective data, time lap between blood sampling and filing the questionnaire may influence the validity of data obtained. Therefore, please give more detailed information on the time schedule.
This indeed is a limitation of our study, which we also mention on page 9 of the manuscript. Also, we clarified the Patients and Methods section on page 4: “The questionnaire was sent to the patients along with an informative letter, stating the goal of this study and explicitly giving the patients the option to opt-out, by returning the questionnaire without filling it out.”

Are there any data available on reproducibility of the evaluation of vasomotor symptoms, e.g. repeated filing of the questionnaire?
Sloan et al. (JCO, 2001) describe that comparable questionnaires exhibited consistent and reliable data. We adapted the definitions of hot flashes that were used by Sloan et al. for our questionnaire.

Results
Page 7 line 181: As both, pre- and postmenopausal women were included in this study the high variability of E2 levels is not surprising. Moreover, correlation of E2 levels and hot flashes should be performed after stratification according to menopausal status (Table 3A and 3B). Results in tables S1a and 1b reflect this drop in E2 plasma levels during menopause.
We thank the reviewer for this important and useful comment. We included the menopausal status in our analyses, as is shown in the methods and results section. Of one patient the menopausal status at the moment of blood sampling was uncertain, we therefore decided to exclude that patient from further analyses.

Discussion
Page 8 lines 228f: Again, for this evaluation patients should be stratified according to menopausal status. Is there any difference in the two groups of patients (w and w/o hot flashes prior to tamoxifen)? According to Dorjgochoo et al. (Menopause, 2009) who investigated more than 5,000 breast cancer patients, frequency of hot flashes is highest for patients at the age 45-55 (the age of menopause).
Please see above. It is indeed important to be informed about the menopausal status of the patients. We have included menopausal status in our analyses.

Page 9 lines 237: Please add a short discussion on sample size.
The following sentence was added to the discussion on page 10: “Also, the modest sample size of this retrospective study requires that these results should be interpreted with care. In particular there were only nine pre-menopausal women who reported hot-
flashes prior to tamoxifen treatment. With so few we are unable to assess whether the apparent positive association in post-menopausal women with prior hot flash histories is particular to this group, or extends to all women with hot-flash pre-treatment histories.”

Minor Essential Revisions:
Page 3 line 71: … by cytochrome P450 (CYP) enzyme. Please include abbreviation The abbreviation was included.

Page 8 line 210: …Lorizio et al. have suggested…
The sentence was adjusted as the reviewer indicates.

Page 16 Table 3: Please include (all patients) or (n=115) in the heading Change made as indicated by the reviewer.

**Level of interest:** An article whose findings are important to those with closely related research interests

**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
Reviewer's report

Title: Hot flashes are not predictive for serum concentrations of tamoxifen and its metabolites

Version: 1 Date: 18 July 2013
Reviewer: N. Lynn Henry

Reviewer's report:
The authors report their findings from a cohort of 115 tamoxifen-treated patients evaluating associations between hot flashes, tamoxifen and metabolite levels, and CYP2D6. The identification of a surrogate biomarker for predicting tamoxifen benefit would be clinically useful. The authors evaluated hot flashes as a potential biomarker and were unable to identify it as a useful surrogate in this small patient cohort.

Major Compulsory Revisions:
1. It is difficult to understand the trial design. On page 4 lines 106-108 the authors report that they do not have to obtain Ethics Board approval for studies that involve a simple single questionnaire. They also mention that they have a cross-sectional design with retrospective collection of hot flash information, and that enrolled subjects had serum concentrations of tamoxifen and metabolites determined as part of routine care. However, this seems to be contradicted by their statements on page 5 lines 111-113 about analyzing the serum samples after a period of storage, which would suggest that the samples were not analyzed for routine care. In addition, it is unclear whether the genetics evaluation was done as part of routine care. Finally, page 6 lines 167-169 refer to 132 returned questionnaires, with 13 patients declining further participation. What additional activities were the patients requested to do (other than complete that single questionnaire)? Therefore, it is not obvious why this protocol did not have to undergo IRB approval and why patients didn’t have to provide informed consent. Even if the patients did have tamoxifen metabolites and genotyping run as part of routine clinical care, a considerable amount of information was collected from these patients other than just having them complete a single questionnaire.

We apologize for the confusion we might have caused for this reviewer. We indeed used a single questionnaire and serum samples obtained from patients to analyze tamoxifen and metabolite concentrations in order to optimize and individualize their tamoxifen treatment. The serum samples were analyzed for routine clinical care. To reduce costs, patient samples were kept frozen for some weeks, in order to analyze more patient samples during one HPLC-MS analysis, as is done more often for expensive diagnostic assays in daily clinical practice. This was clarified by removing line 123-124. The phraseology of the returned questionnaires appeared to be a bit unclear, what we meant to say was that patients had the option to return the questionnaire without filling it out, and state that they would not participate, when they did not want to be included in this study, this is in line with the opt-out option described in the Code of Conduct for dealing responsibly with human tissue in the context of health research (Federa, 2011, http://www.federa.org/sites/default/files/digital_version_first_part_code_of_conduct_in_uk_2011_12092012.pdf). Participation in this study only included the single questionnaire, no other activities were requested. To clarify this in the manuscript, we changed the lines on page 4 to: “Retrospectively, these patients were asked whether they would be willing to complete a single, short questionnaire concerning biometric data and the side effects they had experienced. The questionnaire was sent to the patients along with an
informative letter, stating the goal of this study and explicitly giving the patients the option to opt-out, by returning the questionnaire without filling it out.” Additionally, we changed the lines on page 7 to: “These 165 patients received the questionnaire. 33 patients did not respond to the questionnaire that was sent and 13 patients returned the reply form empty, thereby choosing the option to opt-out and not participate in this study. In total, 119 patients returned a filled out questionnaire, of which 115 forms were correctly completed. Six patients were excluded for the following reasons: one patient had an uncertain menopausal status at the moment of blood sampling; one patient was taking medication to relieve menopausal complaints; it turned out that two patients used tamoxifen less than two months at the moment of blood sampling and two patients used tamoxifen for distant metastases for an exceptionally long time (over 6 years). In total, 109 patients (all female, age mean (range) 51 years (22-76)) were enrolled in the study.”

In conclusion, the serum samples were drawn as part of clinical care, the results of the tamoxifen metabolite analysis was discussed with the treating physician and these results were used to optimize the patients’ treatment. Later, the patients were asked to fill out a single, short questionnaire with questions about the experienced hot flashes, where they were explicitly given the option to opt-out, i.e. to not participate. Since the patients only had to fill out a single questionnaire, no formal medical ethical approval was needed.

According to Dutch legislation, no IRB approval is required for the use of anonymized left-over diagnostic human material with an easy opt-out system and coded data. We clarified this in the manuscript by adding the sentence “DNA was isolated from 200 µL serum that was left over from the tamoxifen and metabolite analysis, using…” to page 5 and adding the sentence “The estradiol concentration was measured in the left over serum sample on…..” to page 6.

2. It is a significant design flaw to not have information about whether or not patients are receiving treatment for their hot flashes. This impacts the primary endpoint of the trial (hot flash frequency and severity) as well as the CYP2D6 phenotype.

The reviewer is correct that this might be a potential confounder we therefore checked all patient records in order to determine whether a patient was using medication to relieve menopausal complaints. Only one patient was using such medication (clonidine). We decided to exclude that patient from our analyses.

3. The final statement in the Conclusion is quite strong, given the limited data to support use of tamoxifen metabolites as predictors of tamoxifen benefit. We adjusted the final statement, indicating that concentration measurements might be a promising method in future.

Minor Essential Revisions:
1. In the abstract the authors state “patients with higher estradiol levels reported more severe hot flashes”. This should be lower estradiol levels.

Change made as indicated by the reviewer.

2. What quality assurance methods were used to verify the genotyping data?
There is a very high reported rate of poor DNA quality (18%). Genotyping was performed according to Standard Operating Procedures, using assays which were validated by direct sequencing. In each run, positive and negative controls were included. The large percentage of genotyping failures can be explained by the fact that DNA was isolated from serum, which is a reproducible and validated method for genotyping in our lab, although the yield is low. The median concentration in this series was 2.46 ng/µl (100 µl eluate) (min 0.22 ng/µl; max 9.50 ng/µl). Since tamoxifen and metabolites are analyzed in serum, and we only used left-over diagnostic material for this study, the DNA had to be isolated from serum.

3. In Table 2, what is the difference between median estradiol concentration (whole cohort) and median estradiol concentration (within detection limits), and how many were in the second group.

We added the following sentence to page 8 of the manuscript: “For 70 (64%) samples the analyzed estradiol concentration was below the lower limit of quantification (LLOQ, 43 pmol/L).”

Discretionary Revisions:
Why was a single questionnaire used to collect hot flash data rather than having subjects complete a diary over a week or so?
Since the hot flash data were acquired retrospectively, a diary over a week would not have changed the results. In the questionnaire, the patients could fill out how many hot flashes they experienced during one day or during one week.

Level of interest: An article whose findings are important to those with closely related research interests
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'