Author's response to reviews

Title: Pre-radiotherapy plasma carotenoids and markers of oxidative stress are associated with survival in head and neck squamous cell carcinoma patients: a prospective study

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

We hereby submit the revised manuscript “Pre-radiotherapy plasma carotenoids and markers of oxidative stress are associated with survival in head and neck squamous cell carcinoma patients: a prospective study” by Amrit Kaur Sakhi, Kjell Magne Russnes, Magne Thoresen, Nasser E. Bastani, Anette Karlsen, Sigbjørn Smeland and Rune Blomhoff for publication in BioMed Central Cancer (BMC Cancer).

The answers to the constructive comments by the Reviewers are written at the end of this letter.

We have read the editorial policies of BMC cancer and verify that the manuscript has not been submitted concurrently to any other journals.

On the behalf of corresponding author, Prof. Rune Blomhoff

Yours sincerely,

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**Answers to Reviewers report**

**Reviewer: Hans Christiansen**

1. The details regarding the treatment are included in the “Method” section under sub-section “Selection of patients and controls” in the manuscript. The post operative doses ranged from 50 to 70 Gy. Two patients with tonsil cancers had a debulking tonsillectomy performed and received 70 Gy at the primary tumor area and one also had positive lymph nodes which received 70 Gy. Others had performed debulking laser surgery for laryngeal cancers and received 70 Gy. The radiotherapy treatment...
was CT based, conformal external photon beam therapy. The majority of patients received 5 fractions per week, but two patients with nasopharyngeal carcinomas received 6 fractions per week. One patient had a split course accelerated hyperfractioned regimen as stated in the article text. As stated in the article text, the concomitant chemotherapy was only administered to two patients. The numbers are two small to influence the data. The regimen used was 40 mg/m2 as in the Dahanca 14 protocol. Concomitant chemotherapy was not standard treatment at the Norwegian Radium Hospital at the time of patient inclusion.

2. The target for the study was to include 100 patients. However, accrual rate was less than expected and in 3 years we had included 78 and the study was stopped. The number of controls were taken from the actually planned number of patients. The details regarding the controls are included in the “Method” section under sub-section “Selection of patients and controls” in the manuscript. The controls were selected from a cohort of 356 participants in a validation study conducted at the University of Oslo. Participants were recruited after response to an invitation letter that were sent to a random selection of citizens in the Norwegian capital city and surrounding area. The aims of the validation study were first to validate a new food frequency questionnaire, and secondly to investigate normal ranges of nutrition-related biomarkers measured in blood samples. The distribution of participants intended to reflect the general population and few exclusion criteria were defined. However, participants with self-reported diseases were excluded before the control group for this study was selected. The results from the study by Anette Karlsen are to be published elsewhere. Therefore, we have to cite it as personal communication.
3. The sentence in the first paragraph in the “Results” section has been changed to “Total tocopherols also showed a trend and were lower in patients as compared to healthy controls (p = 0.07).

4. There is difference between the patients treated in the adjuvant situation (post-operative radiotherapy) and the patients treated by primary radiotherapy (only radiotherapy). The Kaplan Meier analyses have shown a log rank p-value for progression-free and overall survivals as 0.03 and 0.005 respectively. This is one of the reasons that the type of treatment was one of the confounding factors in the Cox regression analysis. We have included a new table (Table 4) showing the survival estimates for all the patients, only radiotherapy patients and post-operative radiotherapy patients for progression-free survival, overall survival and loco-regional control.

Reviewer: Matthias Kappler

1. The blood samples of patients were taken only in the heparin tubes. For control samples the blood samples were taken in heparin tubes for the analysis of FRAP, d-ROMs and ascorbic acid. However, for carotenoids and tocopherols, the control blood samples were taken in EDTA blood sampling tubes. Since for carotenoids and tocopherols, there was a difference between the blood sampling tubes we have done an additional experiment to find out if the use of different anticoagulant effects the plasma levels of carotenoids or tocopherols. We found that there was no difference between EDTA or heparin blood sampling tubes for carotenoids. For tocopherols, we found significant difference between EDTA and heparin tubes for alpha-, beta-, and gamma- tocopherols. We have corrected control values for the correction factor before doing any statistical analysis. The patient and control samples were measured with the same methods.
2. The major difference between total FRAP and modified FRAP is that in modified FRAP the uric acid is removed using enzyme uricase. This difference is also explained in the section “Methods” under the sub-section “Analysis”. No, we have not analysed uric acid separately in controls and patients.

3. The p-values in the Figure 1 caption now show the adjusted Cox regression analysis.

4. The results for overall survival are included in the revised manuscript in Tables 5 and 6.

5. The reviewer asks for a two-factor model to investigate whether patients with high level of total carotenoids and high increase in d-ROM have a better survival than patients having a low level of both these factors. We have done this by running a Cox model with these two factors and the interaction between them. Due to a limited number of observations, no other covariates were included in this model. The interaction term turned out non-significant and was removed from the model. Based on the resulting model, the effects asked for by the Reviewer were estimated. We have included this interesting observation in the section “Results” under the sub-section “Changes during radiotherapy” and in the discussion section.

6. The articles are discussed in the manuscript.

**Discretionary Revisions:**

1. No, we have not compared these 24 patients (receiving assisted nutrition fortified with beta-carotene) with other patients. The only thing we found that beta-carotene did not decreased after radiotherapy. However, a sub-group analysis excluding these patients showed a trend for decline in beta-carotene too (p = 0.06).

2. For FRAP change, the p-value was 0.050 after adjusting for confounding factors and is not rounded off.
3. We have mentioned in the text that the hazard rate ratios are to be interpreted as relative risks. There is a kind of tradition for giving these ratios as exposed group vs. controls. In our study, we consider high carotenoid level and high increase in dROM as exposure, and therefore these groups are used as numerators in the ratios, giving ratios below one that is to be interpreted as reduced risk. The overall survival data is also integrated in the paper.

4. We appreciate the Reviewer for this interesting point of view and is included in the discussion.

5. We have adjusted for stage in the calculation for factors 8-iso PGF$_{2\alpha}$ and FRAP in Cox regression analysis.

6. The abbreviation for GGT is changed to Latin in the section “Abbreviations” in the manuscript.

Additionally, we found that confounding factor dROM and FRAP change were wrongly written and are corrected in the revised manuscript. In Cox regression data, some numbers were rounded off wrongly and are corrected in Tables 5 and 6.