Author's response to reviews

Title: Polymorphisms in NFkB, PXR, LXR, interaction with meat, tobacco smoking, NSAID use, and risk of colorectal cancer in a prospective study of Danes

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Version: 3 Date: 24 June 2010

Author's response to reviews: see over
Dear Editor

Thank you very much for the opportunity to respond to the reviewers' comments and revise our manuscript "Polymorphisms in NFkB, PXR, LXR, interaction with meat, tobacco smoking, NSAID use, and risk of colorectal cancer in a prospective study of Danes".

We have revised the paper according to the suggestions from the expert reviewers. Below, we address the point raised by the reviewers and explain how we have dealt with the comments.

We sincerely hope that you will be able to accept the revised manuscript.

Yours sincerely,

Vibeke Andersen

Reviewer's report

Title: Polymorphisms in NFkB, PXR, LXR, interaction with meat, tobacco smoking, NSAID use, and risk of colorectal cancer in a prospective study of Danes

Version: 2 Date: 27 February 2010

Reviewer: Yuanyuan Lu

Reviewer's report:

This manuscript provided valuable information about polymorphisms of transcription factors and lifestyle in association with colon cancer risk. However, before it is found acceptable, the authors should consider the following points:

Major:

1. How did the sub-cohort sample come from the cohort (57053 persons)? Please describe the detailed random method or step. Author should provide a flow chart about subjects selection of this cohort study.

Answer: A flow chart describing the study group is now included as figure 1. The random method of selection of the sub-cohort was completely random. The only thing taken into account was the proportion of the male/female ratio in the sub-cohort was the same as in the case group. We have furthermore included a section about the study design in the discussion.

2. In “Lifestyle variables” author defined the smoking intensity, but in “statistical analysis” smoking status was used which had not been mentioned before. Please explain why you chose smoking status instead of smoking intensity as baseline characteristic?

Answer: We apologize for the inconsistency. Information on smoking was only available at study entry and therefore, we only included smoking status as a variable in the analyses.

3. The conclusion “Del-allele carriers of NFkB -94ins/del were at 3% higher risk pr 25 g meat/day (CI: 0.97-1.08) whereas among homozygous carriers of the wild
type ins-allele, the association was in the opposite direction (IRR pr 25g/day: 0.96, CI: 0.90-1.04, p for interaction= 0.03)” was not exact, though p for interaction= 0.03, IRR pr 25g/day: 0.96, CI: 0.90-1.04 was not significant. 

Answer: in the interaction analysis, we calculate the slopes of two curves describing the relationship between meat intake and CRC risk for homozygous carriers of the wildtype allele and for variant alleles, respectively. Neither of the slopes is statistically significantly different from 0, as indicted by the confidence intervals. However, the two slopes are statistically significantly different from each other as indicated by the p-value for the interaction (p=0.03). We therefore conclude that we do see interaction between genotype and meat intake in relation to CRC risk.

Minor:

1. Interaction about NSAID use and the studied genotype NFkB -94ins/del should be discussed in the discussion part because of the significant CI (1.05-2.32).

Answer: There is no interaction between NSAID use and the NFkB1 polymorphism. In fact, the risk estimates in absence or presence of NSAID are very similar. Thus variant allele carriers are at 43 or 56 % increased risk of CRC in the absence or presence of NSAID use, respectively.

Reviewer: Kiyonori Kuriki
Reviewer’s report:

Dear Authors;

In a nested-case-control study within a Danish prospective cohort study, the authors examined interactions between the six gene polymorphisms of transcription factors and nuclear receptors, i.e., nuclear factor kappa-B (NF#B), pregnane X receptor (PXR) and liver X receptor (LXR-beta), and heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs) derived from red and processed meat, and tobacco smoking, for risk of colorectal cancer. The findings, however, were not clearly shown and appropriately discussed.

Major comment

1. Numbers of the study subjects were not clearly shown in the text (including Abstract) and Tables, so that the data and the findings should be checked. In Method section 359 cases and 765 sub-cohort members were selected, as well as the data in your previous study regarding the MDR1 gene polymorphism (reference No. 20; BMC Cancer 2009:9:407). The findings in the current study, however, were based on 383 cases and 763 sub-cohort members and shown in Abstract, Result section, and all of Tables. Why?

Answer: In total, 405 cases (184 women and 221 men) of colorectal cancer were diagnosed among the cohort members between 1994 and 2003 and registered in the files of the nationwide Danish Cancer Registry [46]. Within the cohort we defined a sub-cohort sample including 368 women and 442 men who were randomly selected. Cases and the sub-cohort sample were frequency-matched on gender. Blood samples were available for 397 cases and 800 sub-cohort members. All information on genotypes and lifestyle factors was available for 378 cases and 756 sub-cohort members who were included in the statistical analyses. In the different published studies, we have included all study cohort members for whom all genotype determinations and lifestyle information was available, and thus differences in success in genotyping results in differences in actual numbers.
cases and controls in the different studies. We now include a flow chart explaining the current study group as suggested by the first reviewer.

2. Family history of colorectal cancer is one of important risk factors for colorectal cancer. This reviewer strongly suggests that the risk should be also adjusted for this factor.

Answer: The prospective ‘Diet, Cancer and Health study’ was not specifically aimed at studying CRC but all cancers. Therefore, the questionnaire only included a question about whether any first relatives had received a cancer diagnosis, but not the cancer type. We are therefore unable to adjust for family history of colorectal cancer, although we are fully aware that this is an important risk factor.

3. As one of potential confounding factors, moreover, the risk should be adjusted for physical activity (or habitual exercise), even if this factor was not related to the risk in this population. In the second expert report based on World Cancer Research Fund/American Institute for Cancer Research, “physical activity” is suggested as a convincing protective factor for the risk. In general, therefore, the majority of readers believe that “physical activity (or habitual exercise)” is related to intestinal active-movement, appropriate defecation and shorter bowel transit time of xenobiotic compounds along with dietary fiber.

Answer: All models are now adjusted for status of HRT (women only), smoking status, alcohol intake, dietary fibres, red meat, BMI, physical activity and NSAID use. We have not adjusted for total energy as suggested. If we adjusting for total energy we will end up doing a substitution analyses, because the model includes red meat and dietary fibres, and this is not the focus of the article. Secondly total energy is not a potential confounder for CRC, and therefore there a no reason to adjust for it. Also if we adjusted we will decrease the power in the study and it would only contribute with noise in the models.

4. Furthermore, the risk should be adjusted for total energy intake as one of confounding factors.

Answer: please see bullet point 3.

5. To facilitate understanding by readers, please kindly state the validity (e.g., Spearman’s rank correlation coefficients between diet records and your food frequency questionnaire) for consumption of red and processed meat, dietary fiber and total energy intake in Method section.

Answer: The requested information is now added in the methods section

6. There were some issues in each Table. In Table 2, were the combined genotypes of the six polymorphisms (e.g., ID + DD) included to calculate the p-values of each interaction? In footnotes “B” in Tables 2, 3 and 5, were age and sex not included? Were the risk not adjusted for age and sex, in Table 4? What was “Crude” meant in Table 5?

Answer: we have modified the footnotes of the tables. We now present two risk estimates, one which is adjusted for age and sex only, and one, which in addition is adjusted for status of HRT (women only), smoking status, alcohol intake, dietary fibres, red meat, BMI, physical activity and NSAID use.

7. Why was not the risk adjusted for use of non-steroidal anti-inflammatory drugs (NSAIDs) in Tables 2, 3, 4 and 5? In Method section and the text, NSAIDs use was described as one of confounding factors.

Answer: we now adjust for NSAID as stated in bullet point 3

8. In the text and Table 3, this reviewer suggests that the risks and the interactions should be categorized and shown by low, middle and high intakes of
red and processed meat consumption, but not “intake of additionally 25 g red and processed meat”.
Answer: we now include a table (table 4) showing tertiles of red and processed meat and the NFkB1 polymorphism as requested.
9. In Table 3, the risks for the two genotypes of the NFkB -94ins/del gene according to red and processed meat intake were not significantly increased or decreased, and the values were almost 1.00. Therefore, the significant interaction might be by a chance.
Answer: The test in table 3 is a test comparing two slopes for cancer risk dependent on intake of red and processed meat for homozygous wildtype carriers and for variant allele carriers, respectively. The risk is close to one when looking at an increase of 25 g of red and processed meat, but when looking at an intake of additionally 100 g red and processed meat the IRR would be 0.89 and 1.13. The p-value of 0.03 is indeed an estimate of the risk of chance finding.
10. To facilitate understanding by readers, in the text, please kindly illustrate what were differences between your previous (reference No. 20) and the current study. Considering contents in Background and Discussion sections, the MDR1 gene seems to be multiply related to the NFkB, PXR and LXR genes, and all of them may be activated by xenobiotic substances, such as HCAs and PAHs. In the current study, the NFkB gene might be just liked to the MDR1 gene. This reviewer, therefore, is wondering the following issue; “what was a new finding?” or “why was not the MDR1 gene included in the current study?”.
Answer: Both NFkB1, PXR and LXR are known to regulate MDR1, but the genes are not genetically linked. We have assessed whether there is gene-gene interaction between the studied genes and MDR1 and found additive, but no synergistic, effects as described in results.
11. Conclusion section should be clearly mentioned. For the risk, why was the NFkB gene polymorphism related to xenobiotic substances derived from red and processed meat, but not tobacco smoking. Likewise dietary intake of red and processed meat, oxidative stress and reactive oxygen spices are also generated by tobacco smoking. Therefore, the data related to fatty meat (i.e., meat rich in fat, but not hem-iron) and “smoking intensity (as described in Method section)” should be shown in the text.
Answer: This subject is now discussed thoughtfully in the discussion. We were not able to include analyses on “fatty meat” as the exposure of fatty meat was not collected in the database. Likewise, we have not been able to find relevant information on intake of fatty meat in European countries in general. For “smoking intensity” we refer to bullet 2 in the answer to the comments by Yuanyuan Lu.
12. Please introduce how high is dietary intake of red and processed meat and tobacco smoking in European countries and the World (in Discussion section). How about was fatty meat intake?
Answer: Data on exposure for smoke and red meat have been added and discussed in the discussion section. We were able to find relevant information on intake of fatty meat in European countries in general.

Minor comment
1. In Abstract, the following sentences were confused; variant allele carriers …, whereas “variant” allele carriers … (p=0.03).
Answer: The mistake has now been corrected.

2. In Table 4, “smoking” should be shown as “smoking status”. Why was “smoking status” included in the footnote A? What was “fully” meant in footnote B?
   Answer: The footnotes have been carefully revised.

3. In Conclusion section, “NFkB polymorphism” was shown as “the NFkB -94ins/del gene polymorphism”. What was “fully” meant in footnote B?
   Answer: “NFkB polymorphism” has been substituted for “the NFkB -94ins/del gene polymorphism”.

Reviewer: Jing Shen
Reviewer’s report:
This is a case-cohort study nested in the prospective population-based Danish Diet, Cancer and Health study. Several well selected genes and polymorphisms were investigated for the associations with colorectal cancer (CRC) risk and the potential interactions with smoking, meat consumption, and NSAID use. The authors found that carriers of the del-allele of NFkB -94ins/del polymorphism were at statistically significantly higher risk of CRC than the homozygous wildtype carriers. This result was acceptable. But from another result to conclude that “there was interaction between the polymorphism and intake of red and processed meat” was not convincing. Although the p-value for interaction between genotype and red and processed meat was significant (p=0.03), the IRR (1.03, 95%CI: 0.97-1.08) was much smaller than carrying genotyping (ID and DD) alone (IRR=1.45, 95%CI: 1.10-1.91). How to explain this interaction? Not only the p value but also the IRR were important to indicate potential interactions. Therefore, the major problem of the current study was the misunderstanding and explanation of the results that should be adjusted and discussed based on revised results. Title also need changed because of no interaction found.

Answer: in the interaction analysis, we calculate the slopes of two curves describing the relationship between meat intake and CRC risk for homozygous carriers of the wildtype allele and for variant alleles, respectively. Neither of the slopes is statistically significantly different from 0, as indicated by the confidence intervals. However, the two slopes are statistically significantly different from each other as indicated by the p-value for the interaction (p=0.03). We therefore conclude that we do see interaction between genotype and meat intake in relation to CRC risk.

Minor comments:
1) Genotyping section: please explain how the “known genotype controls” was obtained (sequence results or other methods? From cancer cell lines or human samples?)? How many kinds of “known genotype controls” were used in the current study (three different genotypes for each polymorphism were included or not)? More information about the 10% duplicated samples was needed (how many duplicated samples were detected? What is their genotyping distribution? Is that consistent with overall genotyping results?). Please introduce some details of the method used for measuring NFkB -94ins/del polymorphism because this polymorphism is the positive finding in the current study.
Answer: details of NFkB1 genotyping are now included in methods. It is now clearly stated that samples of all three genotypes were identified in pilot genotyping, and these were included as controls in subsequent runs. A random 12% of the cohort was genotyped again and 100% identity was found. The genotype distribution was similar to what was previously found. The success rate in genotyping was slightly lower than for the first genotyping run because failed genotypes were not reanalysed.

2) Statistical analysis: please explain what is the “unweighted” analysis?
Answer: When doing the statistical analyses you can choose between different weighting methods. The different in the models is how the cases are handled at time of failure and the weighted of the sub-cohort. In this article we have chosen to use the unweighted methods, and this mean that the sub-cohort members have the weight of 1 at time of failure, and 0 in at all other times. This is described in more details in the Barlow et. al, 1999.

3) No explanation for the results of Table 1. It seems no significant difference observed for those risk factors of CRC between cases and controls. Is this consistent with results from all samples of the cohort? If not, there may be selection bias exist. Please discuss the potential impact of the bias for the current conclusion.
Answer: Comparing the sub-cohort with the descriptive table from previous published articles of the whole cohort looking at CRC, we find only very small differences in the values. Therefore we don’t think selection bias has a potential impact of the conclusions in the given study. We have included a paragraph discussing the study design

4) Results section: Although the details for the PXR and LXR haplotypes may not be necessarily showed, it is better to describe certain basic information obtained from haplotype study, such as how many different haplotypes observed? What is the common (top three) haplotypes constitute of? What is the frequency of the top haplotypes?
Answer: Haplotype analyses of the PXR gene showed eight different haplotypes. We have now added detailed data on the most common haplotypes suggested.

5) Results section: the description of “Del-allele carriers of NFkB -94ins/del were at 3% higher risk pr 25 g meat/day (CI: 0.97-1.08) whereas among homozygous carriers of the wild type ins-allele, the association was in the opposite direction (IRR pr 25g/day: 0.96, CI: 0.90-1.04, p for interaction= 0.03)” was confusing. The reference group of the comparison was unclear. Please describe it in details with a simple but clear way.
Answer: in the interaction analysis, we calculate the slopes of two curves describing the relationship between meat intake and CRC risk for homozygous carriers of the wildtype allele and for variant alleles, respectively. Neither of the slopes are statistically significantly different from 0, as indicted by the confidence intervals. However, the two slopes are statistically significantly different from each other as indicated by the p-value for the interaction (p=0.03). We therefore conclude that we do found interaction between genotype and meat intake in relation to CRC risk.