Author's response to reviews

Title: Is the impact of methylmercury and n-3 polyunsaturated long chain fatty acids on myocardial infarction risk modified by genetic polymorphisms in glutathione-related genes? A case-control study.

Authors:

Karin S Engström (karin.engstrom@med.lu.se)
Maria Wennberg (maria.wennberg@envmed.umu.se)
Ulf Strömberg (ulf.stromberg@med.lu.se)
Ingvar A Bergdahl (ingvar.bergdahl@envmed.umu.se)
Göran Hallmans (goran.hallmans@nutrires.umu.se)
Jan-Håkan Jansson (janhakan.jansson@vll.se)
Thomas Lundh (thomas.lundh@med.lu.se)
Margareta Norberg (margareta.norberg@epiph.umu.se)
Gerda Rentschler (gerda.rentschler@med.lu.se)
Bengt Vessby (bengt.vessby@pubcare.uu.se)
Staffan Skerfving (staffan.skerfving@med.lu.se)
Karin Broberg (karin.broberg@med.lu.se)

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Author's response to reviews: see over
Dear Editorial Team at Environmental Health,

Thank you for the valuable comments from the reviewers. We hope that our revised manuscript will be satisfying for publication in Environmental Health. Changes in the manuscript are showed by the “tracked changes” function.

Please find below a point-by-point response to the answers to the comments from the editorial team and the reviewers.

Best regards,
Karin Engström

Comments from the Editorial Team:

On the title page, the title should not be a question and must include the study design, for example "A versus B in the treatment of C: a randomized controlled trial X is a risk factor for Y: a case control study". Please remember also to correct the title on the server record.

The title has now been changed to “Evaluation of the impact of genetic polymorphisms in glutathione-related genes on the association between methylmercury or n-3 polyunsaturated long chain fatty acids and risk of myocardial infarction: a case-control study.”

Each affiliation should be written in full, in the following format: department, institute, city, and country. This may mean repeating institutional addresses if the authors are from different departments within an institution.

This has now been changed.

In the Abstract, please avoid the use of abbreviations.

The abbreviations have now been removed. However, since names of genes are generally used in abbreviated form we would like to keep those abbreviations (GCLC, GCLM, GSTP1), if possible.
References of 3 or more sequential numbers should be written as [18-20].

**This has now been changed.**

The heading of the section after the Conclusions should read List of Abbreviations and be listed as abbreviation:term in sentence format and separating the pairs with semi-colons.

**This has now been changed.**

At the end of the Authors' contributions please insert "All authors read and approved the final manuscript."

**This has now been inserted.**

The heading of the next section should read Acknowledgements.

**This has now been changed.**

All references listed in this section must be cited in the text; only 1-28 are cited, but there are 29 references.

**Reference nr 29 was in Table 2. This reference has been moved to the Material and methods section (page 7, below “Genotype analyses”) “Genotype frequencies as well as the ID numbers (rs numbers [26]) for the polymorphisms are presented in Table 2.”.**

The reference section should be formatted as number, period,space (not tab) and reference.

**This has now been changed.**

Are the page numbers missing from reference 5?

**This article has now been published and the correct page numbers have been added.**

All authors must be listed (see reference #26).

**All authors are now listed for reference #27 (formerly reference #26).**

All horizontal lines in the tables must be visible.

**All horizontal lines in the tables are now visible.**

**Reviewer:** Claudia Gundacker

**Reviewer's report:**
Engström and colleagues report that genetic polymorphisms in GSH related genes (GSTP1, GCL) have no significant impact on the risk of first ever myocardial infarction (MI) in a Swedish population (N=1027). The issue of
genetically determined susceptibility towards disease is very important. This specific gene-environment interaction in relation to MI risk has not been investigated so far. The manuscript is well-written, the methods are appropriate and adequately described. The data are sound and well controlled. Before publication, the manuscript needs some revision (minor essential revisions).

Authors are stating that carriers of the variant alleles, i.e., GCLM-588 TT genotype and joint GSTP1 polymorphisms have lower risk of MI compared to reference genotype. Statistical comparisons show that the observed effects are non-significant. This is explained by the small amount of TT carriers in this sample. Indeed, this may have increased the probability for false-negative results. It would be desirable to provide the number of individuals present in subgroups (Fig. 2 and 3). The reader has to deduce from Table 2 that about 40 study participants are homozygous carriers of the T allele. The distribution among tertiles remains unknown.

The distribution in the tertiles of individuals with different genotypes has now been added to the figures. Explanatory footnotes have been added in the figure and to the figure legends.

It is not explained why authors stratify ORs into tertiles (PUFA- and Ery-Hg-levels, respectively).

This manuscript is an extension of another paper (Wennberg et al. Am J Clin Nutr). The present manuscript is based on the same study cohort and to a large extent the same individuals as in the study by Wennberg et al. For simplifying the reading, we wanted to use P-EPA+DHA and Ery-Hg in a similar fashion as in Wennberg et al, where these variables were divided into tertiles. We can see that this is not satisfactorily described in the manuscript. We have thus added the following in the materials and methods section (Below “Statistical analysis”, in the end of page 7) “Since this study is an extension of the study by Wennberg et al [5], P-EPA+DHA and Ery-Hg were evaluated in a similar fashion - divided into tertiles - as in Wennberg et al [5].”

By stratifying the genetic analyses into tertiles of P-DHA+EPA and Ery-Hg, we got the possibility to evaluate the genetic impact at different levels of P-DHA+EPA and Ery-Hg in order to see if the effects differed at different exposures.

Authors state that „the GCLM-588 TT genotype had a lower risk relative to the CC genotype in each P-EPA+DHA or Ery-Hg tertile ...“ This is not true regarding Ery-Hg in tertile 2.

This is correct. We have now changed the text to “the GCLM-588 TT genotype had a lower risk relative to the CC genotype in all P-EPA+DHA or Ery-Hg tertiles except for one (intermediate tertile for Ery-Hg)” (in the results section of the abstract, as well as in the results (end of page 9, beginning of page 10) and discussion (end of page 10) in the main text). In the abstract, the definition of which tertile that was the exception was not included due to lack of space.
Ideally ORs and confidence intervals are presented in a table. It is hard to discern from Fig. 2 and 3 what the exact ORs are. Only the data for GCLM-588 and GSTP1-105/GSTP1-114 genotypes should be divided into tertiles, if at all.

The figures give a graphical overview of the results, but we agree that it is difficult to read what the exact OR:s and CI:s are. We have now added values for ORs and CI:s in the figures.

I miss a rationale for evaluating the effects of Hg-related susceptibility genes in relation to PUFA levels.

The rationale for evaluating the genetic impact on the association between P-EPA+DHA and MI risk was to evaluate the genetic impact on the association between MeHg metabolism and MI risk. This has been clarified by adding the following in the manuscript: (Statistical analysis section, page 8, line 10) “Genetic factors may have an impact on MeHg metabolism, resulting in an individual variation regarding retention and elimination of MeHg at similar exposure levels. Differences in retention/elimination lead to differences in the amount of MeHg that has the opportunity to target vulnerable organs and cause toxic effects that may increase the risk of MI. In order to evaluate this, P-EPA+DHA can be used as a proxy for intake of MeHg, since P-EPA+DHA correlates with the body burden of MeHg.”

Authors say that in one previous study, the GCLM-588 TT genotype demonstrated the highest levels of Ery-Hg, and that it displayed the lowest risk of MI in each P-EPA+DHA tertile in this study. It remains unclear whether the GCLM-588 TT genotype has the highest levels of Ery-Hg also in this study (are the study groups the same?).

We did not include the levels of Hg stratified for each genotype in this paper, since we felt that these results would require much more space in the manuscript. To note however, the GCLM TT carriers among the controls displayed the highest Ery-Hg levels. We have now added as follows to the results section: “Among the controls, the GCLM TT carriers demonstrated a mean Ery-Hg level of 5.4 µg/l compared to 4.9 µg/l among the GCLM CC+CT carriers (p-value = 0.65, ANOVA) (end of the results section, page 10). “ as well as in the discussion “The GCLM-588 TT genotype had the highest levels of Ery-Hg also among the controls in this study, although this was not significant. (beginning of page 11)” The study groups were not the same in the two studies (see question number 1 from the other reviewer for more detailed information).

Engström and colleagues are concluding „this suggests that Ery-Hg has a true effect on MI risk and is not only reflecting fish intake“. This statement is not traceable.

This statement has now been removed.
Reviewer: Gunnar Nordberg

Reviewer's report:
Review of Ms MS ID : 1080832050490233 by Engstrom et al

Comments for authors and editor:
This is an interesting article dealing with mercury erythrocyte levels, n-3 polyunsaturated long chain fatty acids and myocardial infarction (MI) among cases and controls in North Sweden. However, it seems that the exposure to methyl mercury in this population group was not high enough to give rise to any impact on MI risk and it is therefore not surprising that the authors did not find any statistically significant modifying effect of genetic polymorphism in glutathione related genes. The observations of relationships (not statistically significant) between genotype and MI may still be of sufficient interest for publication provided the same observations have not previously been published in ref 7 by the same authors. The present reviewer would appreciate to receive the author’s comments on how the observations in the present manuscript differ from those published in ref 7.

These observations of relationships between genotype, Hg and MI have not been published previously in Ref 7 (Schläwicke Engström et al. 2008).

The paper published in ref 7 investigated if genetic polymorphisms modified the metabolism of MeHg. This was evaluated by examining the genetic impact on the association between P-EPA+DHA (proxy for fish intake) and Ery-Hg.

The present paper investigated if genetic polymorphisms (some of them overlapping with the polymorphisms investigated in ref 7) modified the relationship between MeHg and the risk of MI. This was evaluated by examining the genetic impact on the association between Ery-Hg or P-EPA+DHA and risk of MI.

These two study populations did not consist of the same individuals, although they were from the same part of Sweden. The study subjects in Schläwicke Engström et al. 2008 were chosen from Västerbotten Intervention program cohort based in their fish consumption and no cases for MI or other cardiovascular diseases were included.

The study population for the present paper was derived from the Northern Sweden Health and Disease Study, which consists of three subcohorts, among these the Västerbotten Intervention program cohort. The study subjects were chosen according to being a case for first ever myocardial infarction. One or two matched controls were chosen for each case.

The levels of P-EPA+DHA and Ery-Hg were similar in both studies.

This issue now been clarified in the manuscript (Introduction, middle of page 5): “In our previous study [7], we evaluated the genetic impact on MeHg metabolism. This study did not include any MI cases and consisted of different study subjects than the present study.”

Since there is no impact by mercury on MI at the dose level studied, it is not
possible to relate such absent impact to any other observation. The present reviewer therefore suggests that the title of the paper be modified to remove this notion. A suggestion for a suitable title would be:”MI risk, ery-Hg, n3polyunsat long fatty acids and genetic polymorphism in glutathione-related genes – a case-control study.” But other titles may be equally adequate and the authors should feel free to modify their title as they wish.

We agree with the reviewers’ comment. The title has now been changed to “Evaluation of the impact of genetic polymorphisms in glutathione-related genes on the association between methylmercury or n-3 polyunsaturated long chain fatty acids and risk of myocardial infarction: a case-control study.”

Abstract:
The abstract should be modified to reflect the findings. In the conclusions section of the abstract, the word statistically should be added. “No statistically significant genetic modifying effects…….”

This has now been added.

The words “is necessary” are repeated twice in the last sentence, please remove.

This has now been removed.

Introduction:
The last paragraph of the introduction (page 5) should be modified to say that this study examined possible relationships between MI and the studied biomarkers without assuming an impact of MeHg on MI risk.

The reason to the assumption in the introduction regarding an impact on MeHg on MI risk is because this study is based on an earlier study that showed an impact of Ery-Hg on MI risk. This has now been added in the manuscript (Introduction, end of page 5): “The present study is an extension of a study by Wennberg et al [5], which evaluated the impact of P-EPA+DHA and Ery-Hg on MI risk in this study population. The study by Wennberg et al. found that Ery-Hg was statistically significantly associated with a decreased risk of MI, while EPA+DHA in plasma indicated a decreased risk of MI (not statistically significant). ”

We have changed the last paragraph to (introduction, end of page 5): The aim of the present study was to elucidate whether polymorphisms in genes potentially involved in MeHg metabolism and/or antioxidant defence can modify the association between EPA+DHA or Ery-Hg and MI risk. The effect of erythrocyte-Se (Ery-Se) on MI risk was also considered.

Methods:
Page 5: The study population should be better described and it should not be assumed that the reader knows the information in ref 5 (not complete in ref list – has it been published?).
It is true that the part describing the study population is light. We have added some more information in the “Study population” section (page 6), including additional information about the study cohorts, exclusion criteria, and classification of smoking habits. This can easiest be seen by the tracked changes function.

Ref 5 (Wennberg et al. Am J Clin Nutr) has now been published (it was in press at the time of submitting the first version of the present manuscript).

The same is true for the description of measurements on page 6.

We have added more information about which methods that were used for measuring APOA1 and APOB as well as selenium (In the beginning of page 7): “APOA1 and APOB were measured by immunoturbidimetry, while Ery-Se was measured by inductively coupled plasma mass spectrometry (ICP-MS; Thermo ×7; Thermo Elemental, Winsford, United Kingdom).

More detailed information, such as limit of detection, coefficients of variance, and reference material are thoroughly described in Wennberg et al. [5].”

Results:
In the first sentence of the results section (page 8) the authors state that the risk decreased with ery-Hg, however this decrease is not statistically significant and it is not causal, so it is suggested that this sentence is modified.

In the beginning of the results section we describe the results from the univariate analyses evaluating the impact of Ery-Hg, P-EPA+DHA and statistically significant background variables.
We have made this section clearer by adding the following to the results section (First paragraph of the results section, page 9): “When genotype was not taken into account, P-EPA+DHA indicated a decreasing MI risk (p = 0.15), while Ery-Hg was statistically significantly associated with decreasing MI risk (p = 0.014), p-values for trend, Wald test for univariate analyses for the trichotomized biomarkers).”. These analyses are not the same as in Figures 2A and 3A, where the adjusted analyses are shown, so the references to these figures have now been removed. The p-values for the adjusted analyses were 0.21 for PUFA 0.097 for Ery-Hg.

The statement on top of page 10 that ery-Hg has a true protective effect on MI risk should be modified. As far as the present reviewer understands, this effect is not statistically significant. It is highly likely that this observation is due to some kind of confounding and it is very unlikely (according to the present reviewer) that it would be causal because there is no support for a beneficial effect of mercury from other epidemiological or experimental evidence.

The effect was statistically significant, but we agree that it is unclear if Ery-Hg has a true casual protective effect on MI risk so this sentence has now been removed.
Acknowledgement may need editing

We have edited the title.

Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.
Declaration of competing interests: No competing interests