Author's response to reviews

Title: Effect of n-3 polyunsaturated fatty acid on gene expression of the critical enzymes involved in homocysteine metabolism

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Author's response to reviews: see over
Response to comments

Reviewer: Lydia Afman

Reviewer's report:
Within this manuscript the authors refer to literature which showed that omega-3 FA intake can result in a reduction of homocysteine levels. The authors would like to study the possible underlying mechanism. Therefore, they incubated HepG2 cells with 200 #M of different omega-3 FA and examined the expression of genes encoding enzymes involved in the Hcy metabolism and analyzed the FA concentration in the phospholipids membrane fraction in the cells. The study is quite brief and several additional experiments should be done before any final conclusion can be drawn in studies with cell lines.

Major issues
A major drawback of the study is the use of cell lines, the questions remains whether the cell lines are good indicators of the liver. Furthermore the reason why this liver cell line is chosen is not explained.

Response:
S-Adenosylmethionine (SAM), the principal biological methyl donor, is synthesized from methionine and ATP in a reaction catalyzed by methionine adenosyltransferase (MAT). In mammals, two genes (MAT1A and MAT2A), play an important role in human hepatocellular carcinoma, which facilitate cancer cell growth [1]. Furthermore, gradually increased level of plasma Hcy is a reflection of the degree of liver injury is more sensitive biochemical indicator of liver cirrhosis and liver cancer [2].

Another point of concern is the way the FA were administrated to the cells, this is not clear from the methods.

**Response:** Cell line was treated with fatty acids as described previously [1,2]. These fatty acids were dissolved in ethanol [1,2]. After incubation at 37°C for 24 h, the treatment groups were added with fresh culture media with a final fatty acids concentration (DHA, EPA, ALA, Cayman, USA) of 150 µM.


[2]. Nina Habermann. Fish fatty acids alter markers of apoptosis in colorectal adenoma and adenocarcinoma cell lines but fish consumption has no impact on apoptosis-induction ex vivo. Apoptosis. 2010, 15:621–630

The authors studied the cells after 48 hours and claim “The changes in PL fatty acid composition of cell membrane are likely to account for the changes in mRNA expression of the critical genes involved in methionine metabolism“. However, it is known that FA can activate gene transcription within a few hours via among other the PPARs, which means that you don’t necessarily need incorporation within the cell membrane to obtain such effects. If the transcription is regulated via the PPARs, the genes are likely to contain a PPRE binding site. This is however not studied.

**Response:** That is true that n-3 PUFA can activate the transcription factors such as, PPARs, LXR, or SREBP-1c. The critical genes involved in Hcy metabolism are likely contain PPRE binding site. Therefore, in future study, we will further investigate the potential mechanism by which n-3 PUFA regulate the critical genes expression by using RNA interference technology or trans genetic rats model.

If the FA really result in a reduction in Hcy levels one might expect a reduction in Hcy levels in the cells and if measurable also in the medium. A determination of Hcy levels would be of additional value.

**Response:** Our previous animal study and human study have demonstrated that n-3 PUFA decrease plasma Hcy concentration. Furthermore, n-3 PUFA regulate the
critical gene expression and enzymes involved in Hcy metabolism. The aim of the present study was to examine the effect of n-3 PUFA on gene expression. In future study, we will further explore the role of n-3 PUFA in Hcy metabolism.

**Reviewer: Mark Bouwens**

**Reviewer's report:**

This study describes the effects of 3 types of omega-3 fatty acids on gene expression of several essential enzymes involved in homocysteine metabolism. The authors show data for 6 enzymes; MTHFR, MAT, CSE, SAHH, MTR and CBS, of which only the first three respond to in vitro exposure to the fatty acids EPA, DHA and ALA.

**General remarks:**

Although the findings presented might be interesting for those with closely related research interests, the data shown in the manuscript are limited and the details often lacking.

English can be approved significantly. I advise the authors to have the paper reviewed by a native English speaker.

Use of references is extensive, but sometimes seems not very effective. This should be critically reviewed by the authors.

**Response:** Revised accordingly.

The discussion is extensive; several parts can be greatly reduced in amount of text.

**Response:** we have reduced some parts in discussion section.

**• Major Compulsory Revisions**

- Methods should be given in more detail and clarified at several points. Some immediate questions that I had were;
  - Were antibiotics added to the culture medium and which concentrations?
    **Response:** we did not add antibiotics in the culture medium.
  - What was the diluting agent for the fatty acids? (EtOH?)
Response: Yes, these fatty acids were dissolved in ethanol as described previously [1,2].


[2]. Nina Habermann. Fish fatty acids alter markers of apoptosis in colorectal adenoma and adenocarcinoma cell lines but fish consumption has no impact on apoptosis-induction ex vivo. Apoptosis. 2010, 15:621–630

Did controls also contain this diluting agent?

Response: Yes. Controls were exposed to an equal concentration of ethanol to that in the fatty acid exposed samples.

Was RNA quantified and were all samples comparable in used amount for cDNA synthesis?

Response: Yes. RNA was quantified with Nanodrop (Peqlab, Erlangen, Germany) and the RNA integrity number (RIN) was measured with Bioanalyzer (Agilent, Böblingen, Germany). No RNA was used with a RIN below 8.5.

Figure 2 has no error bars, while it is described that there were 3 replicated in three experiments and that each gene was duplicated twice. What is the n shown in this figure?

Response: We have corrected the figures.

An important discussion points is lacking and could be addressed in the discussion section; does the amount of RNA of an enzyme correlate with it activity and functioning?

Response: Yes, we have addressed this issue in discussion section. Because HHcy is caused partly by genetic factors, including polymorphisms of genes encoding enzymes involved in Hcy metabolism, such as MTHFR, MTR, MTRR, and CBS. A common mutation in MTHFR, MAT, and MTR results in a thermolabile variant with reduced activity [1-2]. Regulation on the gene expression of these critical genes using nutrients will be beneficial for their coded enzyme activity.


- The data represented are limited. It would greatly improve this manuscript if data was collected regarding;
  - Enzyme activity
  - Protein expression
  - SAM concentrations
  - Potential transcription factors involved, using a more extensive in silico analysis tool, as started in the discussion.

Response: That is true. The data of the enzyme activity, protein expression and some transcription factors mRNA expression will definitely improve the manuscript. While, our previous animal study also demonstrated that the level of plasma Hcy was significantly decreased with tuna oil (TO); methionine adenosyl transferase (MAT) activity was significantly increased and MAT mRNA expression was significantly upregulated with TO; cystathionine-gamma-lyase mRNA expression in TO was significantly upregulated; however, cystathionine beta-synthase and S-adenosylhomocysteine hydrolases were not significantly changed when compared with control. In present study, we try to examine the effect of n-3 PUFA on the gene expression of mRNA of the critical gene. In future research, we hope to explore the potential mechanism by which n-3 PUFA regulate the expression of the gene mRNA.

• Minor Essential Revisions
- Abstract, methods; sentences are not proper English. Rewrite.

Response: Revised accordingly.
- Abstract, line 15; three groups have not be described before.

**Response:** Revised accordingly.

- Abstract line 22-23; Delete last sentence.

**Response:** Revised accordingly.

- Page 3, line 20-22, this needs a proper reference.

**Response:** Revised accordingly.

- Page 4, line 9; phospholipids abbreviation has not been explained yet.

**Response:** Revised accordingly.

- Page 4, line 19; …one report.. = paper from authors themselves. In other words, you suggested…..

**Response:** Revised accordingly.

- Page 5, line 16; must be ‘one ml containing 1x10^5 cells’

**Response:** Revised accordingly.

- Page 5-6, line 22-1; delete sentence ‘The phospholipids….Gas chromatography’

**Response:** Revised accordingly.

- Page 6, line 5; rpm should be converted to rcf

**Response:** Revised accordingly.

- Page 6, line 6; which solvents were used?

**Response:** we have described the determined methods of fatty acids more in details.

- Same line; describe TLC.

**Response:** Revised accordingly.

- Page 6, line 11; this line seems to indicate that the fatty acids were solved in ethanol and that the control does contain ethanol. Make clear in methods.

**Response:** Yes, Controls were exposed to an equal concentration of ethanol to that in the fatty acid exposed samples. We have clarified this the methods.

- Page 6, line 14; reference used is not specific, use other

**Response:** Revised accordingly.

- Page 8, line 7-9; text is not detailed enough, clarify

**Response:** Revised accordingly.

- Page 9, line 5; delete ‘firstly’ is there is no ‘second’
Response: Revised accordingly.
- Page 10, line 7; 12 gram per what time unit?
Response: Revised accordingly.
- Page 13, line 9-11; expand on this and use as results
Response: We have used this information from Mapper as results (Table 4).

- Table 1, last gene; I assume beta-actin is intended, but both name and accession number are incorrect (accession number is for mouse 18S gene).
Correct this.
Response: Revised accordingly.

• Discretionary Revisions
- It is unnecessary to describe the complete comparative Ct method (page 7, line 3-18), this has been done before and often better. You can refer to previous publications.
- Can you give arguments for the choice for 150 microMolar of PUFA, while plasma concentrations of these fatty acids are far lower (more in range of 10 uM)
Response: We used this concentration (150 µM) as described by Colquhoun A. The results of the fatty acids in present study were in percentage (%).

Reviewer: Mahmoud Djalali
Reviewer's report:
Major Compulsory Revisions:
1-Method in abstract is not informative, the groups and enzymes should be defined clearly.
Response: Revised accordingly.
2-Conclusion in abstract, fatty acids had different effects on enzymes expressions, they should be mentioned specifically.

**Response:** Revised accordingly.

3-Page 6: assay of fatty acids should be explained more in details.

**Response:** Revised accordingly.

4-Page 8, line 8 what do you mean with research group?

**Response:** Revised accordingly.

5-The statistical tests are not mentioned specifically in result section.

**Response:** Revised accordingly.

6-In case of doing multiple comparisons, the post hoc test should be mentioned. In case of not doing that it seems to be unacceptable.

**Response:** Revised accordingly.

7-Discussion is the repetition of introduction, it should be revised thoroughly.

**Response:** we have revised the introduction and discussion section accordingly.

**Minor Essential Revisions:**

1-Results in abstract, abbreviations are used. Try to use them in full names for the first time.

**Response:** Revised accordingly.

2-Page 11, line 19 in remethylation pathway.

**Response:** Revised accordingly.

3-Page 5, line 5: the sentence seems to be incomplete.

**Response:** Revised accordingly.

4-Figure 1 legend should be changed, because the effects of n-3 PUFA on all of enzymes were not detected.

**Response:** Revised accordingly.

5-The text should be revised again to improve its english quality.

**Response:** Revised accordingly.

**Discretionary Revisions:**
The labels in table 2 and in figure 2 are not informative; please try to make them more clear (a, b, c).

**Response:** We have revised the related information and made it clearer.