Author’s response to reviews

Title: MTTP-297H Polymorphism Reduced Serum Cholesterol but Increased Risk of Non-Alcoholic Fatty Liver Disease

Authors:

Pi-Jung Hsiao MD (pjhsiao@cc.kmu.edu.tw)
Mei-Yueh Lee MD (lovellee@hotmail.com)
Yeng-Tseng Wang PhD (c00jsw00@kmu.edu.tw)
He-Jiun Jiang MD (960219@gmail.com)
Pi-Chen Lin MS (pichili@kmu.edu.tw)
Yi-Hsin Connie Yang PhD (yihsya@kmu.edu.tw)
Kung-Kai Kuo MD, PhD (kuoksfo@yahoo.com.tw)

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Author’s response to reviews: see over
Dear Tim. Sands, Executive Editor of *BMC Medical Genetics*

E-mail: Tim.Sands@biomedcentral.com

We are very grateful for your processing of our manuscript ID: 4668072091688515 “*MTTP*-297H Polymorphism Reduced Serum Cholesterol but Increased Risk of Non-Alcoholic Fatty Liver Disease”. We would like to express our best regard and sincere thanks to the expert reviewers for their brilliant suggestions. According to the editor advice, we have detailed review our original database and evaluate all the statistics again for this revision. Besides, we also amended our manuscript as the reviewer’s comments as following:

**Response to reviewer 1 (Dr. Ken Sugimoto):**

1. The authors recruited over a thousand subjects in this study, however, there must be gender bias because the ratio of male was about 90%. Can the authors explain the reason and stress the importance of the current findings without sex bias? The authors should add some explanation about this issue or perform the same analyses after changing the population.

   **Ans:** We sincerely appreciate this important suggestion. Our recruited population from the health check-up at the Department of Preventive Medicine at our hospital is always distributed and limited by the male dominant in the factory. Even no previous report has mentioned the MTP function has gender bias, we still perform the multivariate analysis adjusted by sex to reduce the gender bias. Number of the recruited 106 female was enough for further multivariate analysis and there was no sex difference in the distribution of allele frequency or genotypes.

2. The authors recruited the subjects with age 16 to 88 y/o. and mentioned 95% of the recruited subjects were between 25 to 65. The authors should limit the
subjects with age 25 to 65 and reanalyze all the data because this study is genetic one.

**Ans:** We are full of gratitude for your kind and expert suggestion. Before going to univariate and multivariate statistical analysis, our population was tested to have a normal distribution in age item. As the cholesterol homeostasis has been proved to have aging effect, we test the genetic effect of *MTTP* polymorphisms adjusted by age and sex in a cross-sectional population to attempt reducing selection bias. We also get important result shown in supplementary file 2, aging and sex have contributed significant role in the serum lipid homeostasis.

3. The discussion section seems to be too long, so this section can be shorten. The authors cited too many previous reports supporting the authors’ findings especially in the discussion section. The reviewer knows that there are a few papers talking about the significance of the Q297H polymorphism of *MTTP* gene, so the authors seemed to do so. On the other hand, there must be some exaggerations in the discussion section to discuss their findings (the latter part of the second and the sixth paragraph). The authors should make it simpler and just speculate the mechanisms underlying the relationship between the polymorphisms of *MTTP* and the development of NAFLD or low concentration of serum cholesterol.

**Ans:** We are very appreciated for this important recommendation. We reconstructed the discussion, delete some content, add 2 references to discuss the rare Q297H in 2nd paragraph, and add a speculation in 4th paragraph.

2nd paragraph: “In KORA study cohort, carriers with homozygous minor allele of 297Q displayed a decrement for BMI and total cholesterol in females but not in males [22]. The homozygous for 297Q and heterozygous -493GT carriers, reported by
Talmud et al., were found to have higher serum triglyceride level and raising effect on apoB levels [23].”

4th paragraph: “In theory, we speculated MTTP 297 polymorphism may alter binding activity of the MTP with apoB, leading to less secretion of apoB-containing lipoproteins. It may result in lower serum LDL-C, non-HDL-C and more lipid accumulation in liver.”

4. In table 4 & 5, the reviewer is wondering why HOMA-IR was negatively correlated with serum LDL-C or non HDL-C. This might be because the authors exclude the subjects with diabetes and the average of HOMA-IR was within normal limits. The reviewer would like to know the baseline characteristics of the recruited subjects, so the authors should show it by sex as a table.

Ans: We are full of gratitude and honestly agree your excellent opinion. Because the hepatic denovo lipogenesis is provided by serum glucose and hyperinsulinemia (25-30%), it should exclude the diabetics to avoid bias for further analysis of the serum lipid or hepatic steatosis. As our previous data has been published (BMC Med Genet. 2013; 14: 54), the conclusion is “Adipo-IR, rather than HOMA-IR, appears to be a consistent insulin resistance index in the study of NAFLD.” The gender effect has been shown in table 4-6 and additional files 1-2. It was always adjusted in univariate and multivariate analysis, and also well compared in the haplotype analysis by SAS system.
Response to reviewer 2. (Dr. XU LIN)

Q1. For SNP selection, the authors should explain why 'MAF >10%' was used.
The common practice is 'MAF>5%'.

Ans: As your comments, we add a sentence to explain in method.

“To achieve significance from our limited sample size, non-synonymous polymorphisms with minor allele frequency more than 10 % in the Han population were chosen according to the SNP reference in NCBI GenBank website.”

Q2. In the statistical analysis, the authors stated that they used multiple linear regression analysis to analyze the association of MTTP genotypes and serum LDL-C, non-HDL-C, and triglyceride. Maybe the authors should spell out whether the dependent variables were normal distributed variables and what variables were controlled for in the multivariate models.

Ans: We sincerely thank for your suggestion and add more detailed explanation in statistical analysis in method. “The Nonparametric Man-Whitney rank-sum test was used to analyze non-normally distributed variable (serum triglyceride).” We also well explained in footnote of table 3.

Q3. Table 2 an Table 3, the author compared the serum lipid levels and metabolic parameters between GG+GC genotype and CC genotype. I would personally like to see these differences among GG homozygotes, GC heterozygotes and CC homozygotes.

Ans: We fully understand what you have concerned. In statistical analysis by one-way ANOVA for metabolic parameters in these 5 polymorphisms and further multivariate analysis, it will be very much complicated for interpretation. And we have already tried but failed to get ideal results because of sample size limitation. We chose the
non-synonymous polymorphisms because of the functional significance by changing amino acid residue. Therefore, we decided logically using the homozygous minor allele (such CC) versus (GC+GC) as a rule for our study. And we also did all the statistical analysis by experienced expert in univariate and multivariate analysis to confirm our results.

Q4. While reading table 6, there seems to be cases/controls without genotype and clinical data. I suggest that the authors should state clearly these in this table. In addition, the authors adjusted for a list of variables including age, sex, etc, in the multi-variable models. I would like to know whether the ORs without adjustment for these variables are different from the adjusted ones. By definition, confounder has to be associated with the exposure (MTTP) and outcome (NAFLD). Could the authors clarify reasons for such adjustments?

Ans: We designed this study to recruit in a cross-sectional population and detailed evaluate the NAFLD by well-trained, experienced hepatologists. The statistical analysis was also well performed by professional to validate our results. We recruited the variates, such as sex, age, BMI, insulin resistance and MTTP polymorphism, together for analysis because all of these confounders are traditionally risk for NAFLD. In manuscript: “Multiple linear regression analysis was employed using serum LDL-C, non-HDL-C, and triglyceride as a dependent variable, while multiple logistic regression analysis was employed using the presence of NAFLD as a dependent variable to recruit BMI, HOMA-IR, Adipo-IR and the genotypes of MTTP polymorphisms as independent variables based on significance in univariate analyses and regression models.”

Q5: In the titles of Table 4–Table 6, the author named” the risk impact and
interaction of the MTTP genotypes on …”, however, the authors did not present any interactions results for gene-environment or gene-gene.

**Ans:** We collected a cross-sectional population to compare all the confounder in a multivariate analysis model after stepwise validation of the significance by univariate analysis. This could clearly define the risk impact on serum lipid by each confounder in a multiple linear regression analysis. Also, we used SAS system to quantitatively compare the impact on serum lipid and risk of NAFLD by all of the genotype combinations (haplotypes) and also adjusted by age, sex and BMI all the time. It is an objective analysis of the gene-gene interaction of the *MTTP* gene in real world. For the gene-environment interaction, we think a well-designed cohort study is necessary to verify.

Q6: The authors stated that there was strong linkage disequilibrium between -493 promoter G/T and Q297H polymorphisms. Could the effect of the -493 polymorphism found by other authors be in connection with the Q297H polymorphism?

**Ans:** Yes, we have cited references 22, 23 in 2nd paragraph in discussion.

“In KORA study cohort, carriers with homozygous minor allele of 297Q displayed a decrement for BMI and total cholesterol in females but not in males [22]. The homozygous for 297Q and heterozygous -493GT carriers, reported by Talmud et al., were found to have higher serum triglyceride level and raising effect on apoB levels [23].”

Q7. Page 28, the first row: Odds ratio (95% confidence interval) instead of Odds Ratio.

**Ans:** Thanks for your great suggestion. We changed the item in table 6.
Q8: Finally, should the Q297H polymorphism be considered as a causal or additional factor of NAFLD?

Ans: Thanks for your brilliant question. As the MTP functions to determine the hepatic secretion of apoB-containing lipoproteins, we rationally speculate the altered function of MTP by Q297H polymorphism may significantly contribute to a higher risk of NAFLD than that of BMI and insulin resistance. It seems to be as a causal factor by pathophysiological mechanism. However, it needs more molecular evidence.
Response to reviewer 3 (Dr. M. Mahmood Hussain)

We are very grateful for your bright and excellent suggestions after a thorough reading this manuscript. We have detailed reviewed the original database and found the fact: the minor allele frequencies of E98D, I128T, N166S and Q297H are quite different in European and Asian. So we corrected the genotype nomenclature as your suggestion. All previous genotype of D98E and T128I were revised as E98D and I128T according to our allele frequency.

Major revision:

1. There are many spelling, grammatical, and diction errors. It makes it very difficult to interpret and follow. Some of the statements contradict themselves. This document must be extensively edited before publication.

   Ans: Thanks for your kind suggestion. We have revised our English writing by an experienced native English writer again before this submission. We hope it will improve the reader’s understanding.

2. Results (paragraph 1) – The authors mention that there is linkage disequilibrium in the MTTP gene. In the discussion, they state (results paragraph 1), “there is strong linkage disequilibrium among these five polymorphisms (Figure 1)”. There was a high connection between haplotypes (G-493T and T128I) and (D98E and N166S).” Which alleles are linked? -493G with I128 or -493T with T128? D98 with N166 or D98 with 166S. Please be clear.

   Ans: We described the connection in 1st paragraph of results. “All of the genotype distributions were tested in Hardy-Weinberg equilibrium (Table 1). There is strong linkage disequilibrium with allelic association in all pairwise combination (pairwise D’ greater than 0.97) among these five polymorphisms (Figure 1).”
3. Throughout the manuscript, the missense mutations are reversed from the usual nomenclature. Usually, the major allele is listed first and the minor allele should be listed at the end. For example, T128I is I128T in most articles and databases. Only, N166S is listed correctly. Further the format is inconsistent throughout the manuscript. This particularly comes into play in Table 1. The missense mutation is listed as T128I. The genotypes are listed as TT:TC:CC. The CC substitution in DNA corresponds to the threonine, while the TT corresponds to isoleucine. This is confusing in the table. This also comes into play in paragraph 1 of the results. For example, T128I (T:C) implies that the T corresponds to the threonine and the C corresponds to isoleucine. This is not correct. The problem with the nomenclature extends further as it appears that your population has a completely opposite allele distribution than reported in most databases. Please confirm all SNPs and interpretation of results.

4. Results (second paragraph) – Authors state “subjects with CC genotype (98E)”…..The codons for glutamic acid are GAA and GAG. The codons for aspartic acid are GAT and GAC. There is a GAG (glutamic) => GAC (aspartic) transition resulting in a Glu to Asp transition. Thus, this statement is incorrect. Also, the conclusions need to modified to reflect these changes.

Ans: We are very grateful for your bright and excellent suggestions after a thorough reading this manuscript. We have detailed reviewed the original database and found the fact: the minor allele frequency of E98D, I128T, N166S and Q297H has quite different in European and Asian. So we corrected the genotype nomenclature as your suggestion. All previous genotype of D98E and T128I were revised as E98D and I128T according to allele frequency in our population. The genotype distribution was shown in table 1. Genotype of the GG: GT: TT was simplified by number 1: 2: 3. All the genotype combination (haplotype) was according to the sequence of

**Minor Essential Revisions**

1. Abstract (background) – Mutations in MTP is implicated in abetalipoproteinemia, not hypobetalipoproteinemia. One paper (Di Leo et al. 2005 Atherosclerosis) implicated MTP missense mutations in hypobetelipoproteinemia. However, this was not the paper that was cited.  
**Ans:** We agreed with your suggestion and delete the “ hypobetalipoproteinemia” in abstract and background.

2. Abstract (Background) – The author’s state “the precise mechanism linking dyslipidemia and NAFLD may be interactions with MTTP polymorphisms.” The term dyslipidemia is vague and misleading. This term can be used to describe elevated or reduced plasma lipids. Are they both linked to NAFLD? Which one is it? Also, the term “precise mechanisms” implies that MTP mutations are the reason the linkage. There are various other genetic and environmental factors that also affect plasma lipids and NAFLD.

3. Abstract (Conclusion): I would not use the term dyslipidemia. This implies that lipid values are out of range. All averages are within the normal range.  
**Ans:** We really appreciated your brilliant advice. So, we deleted the vague term” dyslipidemia” and used more specified term as “serum lipid” or “apoB-containing lipoproteins” in manuscript. So we revised the background as your hint.  
“Background: Microsomal triglyceride transfer protein (MTP) works to lipidate and assemble the apoB-containing lipoproteins in liver. It closely links up the hepatic secretion of lipid to regulate serum lipid and atherosclerosis. Cases of **MTTP** gene
mutation is characterized by abetalipoproteinemia and remarkable hepatic steatosis or cirrhosis. Several MTTP polymorphisms have been reported relating to metabolic syndrome, hyperlipidemia and steatohepatitis. We supposed the regulation of serum lipids and risk of non-alcoholic fatty liver disease (NAFLD) formation may be modified by individual susceptibility related to the MTTP polymorphisms.”

Conclusion was also revised as” These results evidenced the MTTP polymorphisms could modulate the lipid homeostasis to determine the serum lipids and risk of NAFLD. The MTTP 297H polymorphism interacted with age, insulin resistance and BMI to decrease serum apoB containing lipoproteins (LDL-C and non-HDL-C) but increase the risk of NAFLD formation.”

4. Background (paragraph 1) –MTP mutations are not widely implicated in Hypobetalipoproteinemia. Hypobeta can be removed from the manuscript.

Ans: Yes, we have done as your suggestion. (delete “ hypobetalipoproteinemia”)

5. Why did the authors use the AAI 25112.1 instead of the more commonly used NP_000244.2 accession number for the MTP template? Please correct.

Ans: We repeat the searching by both assays to determine and compared for the MTP proteins. It does show as the same amino acid residues. Therefore, we still used our familial method.

6. Background (paragraph 2) – Author’s wrote “an imbalance of fatty acid homeostasis may contribute to the development of NAFLD, including dietary intake of chylomicrons.” The intestine makes chylomicrons to distribute dietary lipids from the intestine to peripheral tissues. Please revise.

Ans: Chylomicron was corrected by “excess dietary fat intake”
7. Background (paragraph 3) – replace transfer catalytic activity with just transfer activity.

Ans: Yes, we corrected as you suggested. (delete “catalytic”)

8. Throughout the paper, the authors are inconsistent with designations of the polymorphisms. For example, in statistical analysis paragraph one when describing a haplotype, they wrote “promoter -493 GG/D98/T128/S166/Q297H” replace with either Q297 or H297, whichever is correct. This is a consistent problem.

Ans: We add contents to describe our nomenclature for haplotype analysis in method. “Principally, genotype of the GG: GT: TT was simplified as number 1: 2: 3. All the genotype combination (haplotype) was according to the sequence of G-493T/E98D/I128T/N166S/Q297H polymorphisms. Frequency of the most popular genotypes combination, -493GG/E98/I128/S166/Q297H simply indicated as 11132 (GG/GG/TT/GG/GC), was 22.43% as reference control to compare the relative risk of other combinations for metabolic abnormalities.”

9. Several studies have investigated the correlation with plasma lipids and H297Q polymorphisms. None of them are cited. It should be noted they give conflicting results and discussed.

Bohme et al. 2008 Molecular Genetics and Metabolism

Ans: We deeply appreciated your notice. So we cited the suggested references and discussed the similarity and inconsistent results in 2nd paragraph of discussion to emphasize our novel finding.
10. On the same note, the authors report, “Our study is the original report that
carriers of the MTTP 297H were significantly associated with lower
apoB-containing lipoproteins (LDL-C, non-HDL-C) and higher risk of NAFLD after
adjustments for age, sex and insulin resistance.” See above papers.
Ans: Thanks for your notice. We revised our conclusion as following,
“In summary, our study demonstrated that carriers of the MTTP 297H significantly
had lower apoB-containing lipoproteins (LDL-C, non-HDL-C) and greater risk of
NAFLD adjusted by age, sex and insulin resistance. Genotype MTTP 297H may be an
important and independent risk for NAFLD formation (odds ratio 1.68, 1.1-2.6)
followed by BMI and Adipo-IR. Our study is the original report using a predicted
molecular modeling to verify the potential functional alteration for this polymorphism.
This result further evidenced the genetic risk in developing NAFLD.”

11. Discussion (paragraph 3) please revise “proteoglycan binding site to apoB”.
Ans: Delete “proteoglycan” and revised as “The I128T polymorphism, changing from
an uncharged to a polar amino acid, was speculated to confer a change in electrostatic
stability and alter the interactive binding activity of both proteins [28].”

12. Discussion (last paragraph) please revise the sentence “Absent of histologic
diagnosis of NAFLD by liver biopsy would reduce the relevance of genetic effect”.
It is unclear.
Ans: As your suggestion, we revised as “Abdominal ultrasound is generally applied in
relatively large-scaled surveys as a noninvasive and convenient tool to diagnose
NAFLD with acceptable sensitivity and specificity [5]. However, absent of severity
grading of the hepatic steatosis in histology would weaken the clinical relevance of
these genetic effects.”
13. Please revise: “Because of the relatively large protein of MTP and apoB, clinical validation of the functional activities of these MTTP polymorphisms, such as lipid binding or membrane transfer with apoB, are limited.”

**Ans:** Revised our sentence as “Because both MTP and apoB are large proteins to interact in complex, clinical validation of the binding or transfer activity with apoB by these MTTP polymorphisms related changes is quite challenging.”

14. Results (paragraph 4) – Authors state “(P= 0.0168, not shown in supplementary 2)”. Please show data both the ratio AST/ALT and the fatty liver formation since you are stating the results and giving a significant p-value.

**Ans:** We revised our supplementary table to add odds ratio of NAFLD with 95% confidence interval. Also, all the AST/ALT was corrected as AST and ALT in manuscript.

**Discretionary Revisions**

1. Results paragraph 5: Remove the sentence “aging has also been found to raise serum cholesterol and non-HDL-C a little.” It is out of place.

**Ans:** We agreed with your opinion to delete.

2. Discussion – Many papers report an association between -493 G/T and plasma lipids. It is the most characterized polymorphisms and shows the strongest correlations in the literature. Interestingly, the authors did not find a statistical correlation. It would be beneficial to point this out and discuss.

**Ans:** We are fully confused by these inconsistent results of the previous reports. So we designed this study to recruit both the promoter and non-synonymous polymorphisms to compare with confounders in a well-designed analysis. Then, it may demonstrate a
real effect of the gene-gene and gene-environment. We cited previous report and discuss in detail in 2nd paragraph of discussion.

All of these revisions were well-checked by authors and edited by native English writer. Any assistance or suggestions to make this manuscript more acceptable for publication is still highly appreciated. We look forward to your favorable consideration.

Sincerely yours,

Kung-Kai Kuo, MD, PhD.

100 Tzyou 1st Rd, Kaohsiung 807, Taiwan
Tel: 886-7-3121101 ext 7651; Fax: 886-7-3122810
E-mail: kuoksfo@yahoo.com.tw

We deeply