Author's response to reviews

Title: ANGPTL4 variants E40K and T266M are associated with lower fasting triglyceride levels in Non-Hispanic White Americans from the Look AHEAD Clinical Trial

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Author's response to reviews: see over
ANGPTL4 variants E40K and T266M are associated with lower fasting triglyceride levels in Non-Hispanic White Americans from the Look AHEAD Clinical Trial Melissa C Smart-Halajko, Alyson Kelley-Hedgepeth, Maria Claudia Montefusco, Jackie A Cooper, Alan Kopin, Jeanne McCaffrey, Ashok Balasubramanyam, Henry J Pownall, David M Nathan, Inga Peter, Philippa J Talmud and Gordon S Huggins

Dear Dr. Bluher,

We would like to thank the reviewers for contributing their time, expertise, and attention to our manuscript. The reviewers identified important areas of our manuscript that required modification and revision. After extensive consideration of each criticism, we have performed new analyses and modified the manuscript. We now submit a revised manuscript for consideration for publication in the *BMC Medical Genetics*.

Please note because we have made substantial changes to the manuscript as motivated by Reviewer 2 we now provide the revised manuscript without tracked changes.

Our response to each review comment is listed below.

Sincerely,

Melissa C Smart-Halajko, Ph.D
Gordon Huggins, MD

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Reviewer 1

We thank the reviewer for helpful comments and criticism. We agree the potential interaction of ANGPTL4 genotypes with treatment interventions is an important part of this manuscript. We now provide responses to criticisms below and a revised manuscript.

1) **A power calculation should be added.**

We now provide power calculations. The following sentence has been added to the methods section: “Using a dominant model there was 80% power to detect a 6.9 mg/dl difference in triglycerides between E40K allele carriers and non-carriers at baseline. Using an additive model there was 80% power to detect a difference at baseline of 2.1 mg/dl in triglyceride levels per T266M allele. Both power calculations are based on a 2-sided test at 5% significance level”.

2) **The authors should more appropriate differentiate between effects in patients on specific lipid lowering drugs or different antidiabetic medication.**

We thank the reviewer for emphasizing the importance of medication effects on triglycerides. Unfortunately Look AHEAD does not supply the names of individual lipid lowering and antidiabetic medication use taken by study participants. The dataset provided by Look AHEAD includes the presence or absence of statins, other lipid lowering drugs, any diabetes medication and insulin. Therefore it is not possible to segregate our analysis by specific lipid lowering or antidiabetic medication use. Further, we anticipate that performing an analysis segregated by specific medications will decrease the sample size to an extent that our power to detect an association would be significantly decreased.

3) **Is the association also detectable in subjects without lipid-lowering medication?**

In response to both Reviewer 1 and Reviewer 2 points we performed a stratified analysis of subjects not taking a lipid lowering medication. We found that Look AHEAD participants not taking a statin or any other form of lipid lowering drug had the same genotype-triglyceride association as was detected in the full cohort. Please see our response to Point 6 below that further describes the results from our stratified analysis.

4) **Fasting glucose levels differ between the genotype groups. Even this difference is not significant analyses should be adjusted by plasma glucose since the strong interaction with triglyceride levels are known.**

We thank the reviewer for emphasizing the importance of glucose control on triglycerides. The results when glucose is added as a covariate in our analysis are presented below:
<table>
<thead>
<tr>
<th></th>
<th>TT Mean (95% CI)</th>
<th>TM Mean (95% CI)</th>
<th>MM Mean (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n males/ n females</strong></td>
<td>461/500</td>
<td>428/417</td>
<td>93/101</td>
<td>-</td>
</tr>
<tr>
<td><strong>Triglycerides (mg/dl)</strong></td>
<td>173.88 (168.35, 179.58)</td>
<td>164.77 (159.18, 170.55)</td>
<td>154.80 (144.13, 166.26)</td>
<td><strong>0.01</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>EE Mean (95% CI)</th>
<th>EK Mean (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n males/ n females</strong></td>
<td>934/934</td>
<td>40/42</td>
<td>-</td>
</tr>
<tr>
<td><strong>Triglycerides (mg/dl)</strong></td>
<td>169.88 (165.98, 173.88)</td>
<td>144.19 (129.12, 161.01)</td>
<td><strong>0.007</strong></td>
</tr>
</tbody>
</table>

Because glucose levels were not significantly different between genotype groups and because the addition of glucose as a covariate did not alter the genotype-triglyceride association results, we chose to not include glucose as a covariate in the revised manuscript.

5) The regression model used should be tested for overfitting since 13 covariates are included. Have the authors performed a stepwise inclusion?

We performed a stepwise regression for all blood lipid and anthropometric measures. The vast majority of covariates, especially those related to demographic and lifestyle traits, were found to have a significant independent effect. Given the fact that we replicated previously reported genetic associations, it is highly unlikely that our statistical models described a random error associated with overfitting. The final selection of covariates was ultimately determined by the Look AHEAD committee in order to maintain consistency throughout all publications arising from the study and was based on both statistical significance and clinical relevance. This committee had a panel of statisticians and clinicians who approved the selection.

6) Furthermore, a stratified analysis for subjects with and without lipid lowering medication should be performed.

In response to both Reviewer 1 and Reviewer 2 points we performed a stratified analysis of subjects with and without lipid lowering medication. We found that Look AHEAD participants not taking a statin or any other form of lipid lowering drug had the same genotype-triglyceride association as was detected in the full cohort. Interestingly, the association of \textit{ANGPTL4}-E40K with triglyceride levels remained significant in the setting of lipid lowering therapies. However, the association of \textit{ANGPTL4}-T266M with triglycerides was no longer detected.

We have added the following sentence to the result: "The association of E40K and T266M with triglyceride levels remained significant in those individuals not taking a lipid lowering medication. The association of E40K with triglyceride levels was also significant in subjects taking lipid lowering drugs, while the triglyceride association with T266M was not found in the setting of lipid lowering therapy (data not shown).".

We have added to our discussion that there are several considerations that might explain the lack of association of T266M with triglycerides in the setting of lipid lowering therapy. These results may indicate an important drug-genotype interaction. Alternatively these findings may reflect the reduced power to detect an association in this subgroup analysis.
Reviewer 2

We thank the reviewer for helpful criticisms and comments. The changes to the manuscript motivated by these comments are listed below and within the manuscript itself.

**Major Compulsory Revisions:**

1) **The working hypothesis by the authors is not well designed.** Since the authors aim to identify possible association of the ANGPTL4 variants with triglyceride levels (continuous trait), which has been already shown by others in 30,000 non-diabetic individuals, it is not entirely clear why the authors try to correlate in subjects with T2D and what the scientific benefit will be.

The importance of triglyceride levels as an established risk factor of cardiovascular disease risk was recently emphasized in a Scientific Statement from the American Heart Association (Circulation 2011;123: currently on line only). T2D increases triglyceride levels and a lifestyle intervention designed to treat T2D can lower triglyceride levels. Given the important role of triglyceride levels in risk of cardiovascular disease in the setting of T2D we performed this study to determine whether ANGPTL4 genetic variants that regulate triglyceride levels in non-diabetic subjects also modify triglycerides in T2D. The importance of answering this question is the scientific and medical community will gain the knowledge whether the ANGPTL4-mediated effects on triglycerides seen in non-diabetic subjects can be generalized to T2D, and whether genetic effects may increase triglycerides in T2D thereby contributing to the risk of cardiovascular disease. Further, we hypothesized that ANGPTL4 variants would modify the triglyceride response to a lifestyle intervention designed to treat T2D. This hypothesis centers directly on the issue of personalized medicine and whether the genetic background (here ANGPTL4 genotypes) could influence the triglyceride response to treatment (here lifestyle intervention). Our study is the first to directly test the role of a genetic factor on the triglyceride response to a lifestyle intervention. We have modified the Abstract and Introduction to clarify our working hypotheses.

2) **Abstract: There is no clear objective presented. Please clarify this.**

We have modified the Abstract significantly to more clearly state our objective.

3) **Abstract: Please present triglyceride levels as mean ± S.D.**

We now present triglycerides as mean ± S.D in the Abstract.

4) **Abstract: “We have demonstrated a significant association of the functionally compromised ANGPTL4 E40K variant with lower triglyceride levels.” (in what population??? T2D or non-diabetic subjects) Please clarify.**

We now clarify that we demonstrate an association in the Look AHEAD cohort, which is comprised of overweight-obese subjects all with T2D.

5) **Abstract: “In addition, our findings suggest that in type 2 diabetes, T266M may contribute to effects on triglyceride levels demonstrating the role of Angptl4 as a regulator of triglycerides levels in T2D.” (since this is a conclusion please describe how exactly T266M contributes)**
The precise mechanism by which T266M affects triglyceride levels remains a topic of scientific investigation. The fourth paragraph of the discussion section discusses potential mechanisms including a reference to a manuscript by Romeo et al which has demonstrated that non-synonymous mutations in the Angptl4 C-terminal fibrinogen domain (where T266M is located) have been found to compromise protein expression and inhibition of LPL activity.

6) Introduction, last paragraph: Please clarify/justify your working hypothesis why the association of the ANGPTL4 variants with lower triglycerides should be assessed in type 2 diabetic individuals.

We have clarified our working hypothesis as described above.

7) Materials and Methods: Please clarify why the presented numbers of genotyped individuals are different from those presented in the Abstract (2601 vs 2708 individuals).

We thank the reviewer for noting this issue and we have corrected this discrepancy.

8) Materials and Methods: Please be more precise with presentation of the variants and use only E40K and T266M. It is not necessary to mention rs numbers (only once).

We now report the rs numbers only once and we always refer to the two ANGPTL4 variants as E40K or T266M.

9) Materials and Methods: Please describe the ethnic sub cohorts you are analysing.

We now include in the Materials and Methods that we are studying Look AHEAD participants who self-identified their race/ethnicity as non-Hispanic White, African American, or Hispanic.

10) Statistical methods: Please add power calculation.

This was also noted by reviewer 1. We have now provided power calculations. The following sentence has been added to the methods section: “Using a dominant model there was 80% power to detect a 6.9 mg/dl difference in triglycerides between E40K allele carriers and non-carriers at baseline. Using an additive model there was 80% power to detect a difference at baseline of 2.1 mg/dl in triglyceride levels per T266M allele. Both power calculations are based on a 2-sided test at 5% significance level”.

11) Statistical methods: did the authors stratified analysis for subjects with and without lipid lowering medication?

In response to both Reviewer 1 and Reviewer 2 points we performed a stratified analysis of subjects with and without lipid lowering medication. We found that Look AHEAD participants not taking a statin or any other form of lipid lowering drug had the same genotype-triglyceride association as was detected in the full cohort. Interestingly, the association of ANGPTL4-E40K with triglyceride levels remained significant in the setting of lipid lowering therapies. However the association of ANGPTL4-T266M with triglycerides was no longer detected.

We have added the following sentence to the result: “The association of E40K and T266M with triglyceride levels remained significant in those individuals not taking a lipid lowering medication. The association of E40K with triglyceride levels was also significant in
subjects taking lipid lowering drugs, while the triglyceride association with T266M was not found in the setting of lipid lowering therapy (data not shown)."

We have added to our discussion that there are several considerations that might explain the lack of association of T266M with triglycerides in the setting of lipid lowering therapy. These results may indicate an important drug-genotype interaction. Alternatively these findings may reflect the reduced power to detect an association in this subgroup analysis.

12) Results: The two variants are not in high LD. The authors explain this, which is correct in the beginning of the first paragraph of the results. Then this paragraph does not make sense: "Because of the strong LD between the two cSNPs, we examined whether the association of T266M with triglyceride levels merely reflected the presence of E40K. When the K40 carriers were excluded, the association of T266M with triglycerides remained significant in multivariate linear regression using a general additive model of inheritance, with M266 homozygotes having 22.6 mg/dl lower triglycerides compared to TT individuals (p=0.002).” Please clarify this.

The E40K and the T266M variants are in partial linkage disequilibrium (D’=1 and r²=0.05). To correct the conflict identified correctly by the reviewer we have corrected this text to read: “We examined whether the association of T266M with triglyceride levels merely reflected the presence of E40K or whether the effect was also observed independently. When the K40 carriers were excluded, the association of T266M with triglycerides remained significant in multivariate linear regression using an additive model of inheritance, with M266 homozygotes having 22.6 mg/dl lower triglycerides compared to TT individuals (p=0.002) (Table 4)."

13) Table 2: The table present genotype frequencies, not allele frequencies. It is not clear to the reviewer how a minor allele frequency (MAF) can have a 95% CI. Please correct this.

We have removed the 95% CI from Table 2.

14) Please show an additive model for both variants. This has been mixed in Table 2 (dominant mode for E40K, because of small groups I guess; additive mode for T266M).

It was not possible to perform the analysis using an additive model because we did not identify a non-Hispanic White Look AHEAD participant to be homozygous for the E40K variant. Therefore, we performed the comparison of only two genotype groups which is consistent with both dominant and additive models. Furthermore, analysis using the dominant model is supported by the published literature on the ANGPTL4-E40K variant (ATVB 2008;28;2319-2325). We have addressed this important point by now including the actual numbers of KK individuals (none) in Table 2 so the reader can see for themselves.

15) The authors can present a p-value for the dominant model for E40K and explain this.

As we discuss above there were no Look AHEAD participants homozygous for the ANGPTL4 E40K variant, which is why we used the dominant model for our analyses. We have addressed this important point by presenting the number of EE, EK and KK carriers in Table 2.

16) There is a second table 2. Please correct this.
17) **Table 3: Again, no genetic model is mentioned. Please show also the additive mode.**

Table 3 is the analysis of E40K genotype on baseline data. As discussed above the dominant model was used for E40K because we did not identify any non-Hispanic White Look AHEAD participant homozygous for the minor allele. We now indicate in the Table 3 legend that a dominant model was used.

18) **There is a second Table 3, please change that.**

We have corrected this issue.

19) **Figure 1: What about the genotype EK/TT? Please add these data as well.**

We did not detect any Look AHEAD participant with the EK/TT combination. We now include in the legend for Figure 1 a statement that indicates that the EK/TT combination of genotypes was not found.

20) **Discussion: Please re-write the discussion chapter according to the reviewer’s comments (above).**

We have extensively rewritten the discussion incorporating the reviewer’s comments.