# Responses to reviewers

## Referee: 1

* *"First, I would like to congratulate you for the exhaustive and complete research work that has been done for this paper. I think the statistical study is very successful and useful for researchers who have accumulated a large database and need a method with the ability to handle a large number of variables and their interactions. I really liked read in the discussion the negative points of the study, with which I fully agree, and not an attempt to hide the limitations of any scientific work. In retrospect, perhaps it will miss data that clarify the pre-diabetic state, or not, of the patients (HbA1c or C-peptide). I think it would be good for the study know if some patients were taking any medication that could affect the results. Although it is a complicated scientific work to understand for researchers who do not usually use this kind of statistical studies, I believe that is described in a very appropriate and clear manner."*
* We thank the reviewer for these comments. In the PROMISE cohort, we collected medication data from all participants. While most participants took some form of medication over the 6 years, during the times of data collection, many (n=60-103 between the three visits) were on taking lipid and cholesterol controlling medications. However, for those who were taking medications that could influence the results, additional analyses revealed that adjustment for medication use did not substantially influence the associations between the predictor or outcome measures, nor did medication use substantially contribute to GEE model fit compared to models without it (see QIC table). We have included this information in the revised manuscript ( … we can indicate location when we are closer to submission … ).

## Referee: 2

* *"The present paper addresses an important and yet unresolved research question regarding the role of non-esterified fatty acids on the trajectories of metabolic traits of diabetes (insulin sensitivity and beta-cell function). The analysis is based on a cohort study with repeated measures (up to 3 times per individual) over 6 years of follow-up using generalized estimating equations (GEE). This is a well-established method to analyse longitudinal data. The authors in general did a thorough analysis however some major questions related to methods and interpretation remains."*
* We appreciated the reviewer's positive comments and detailed suggestions. In the responses below we try to address each of the comments raised by the reviewer.
* *"After the trajectory analysis they run partial least-squares discriminant analyses (PLS-DA) with groups defined by beta-cell trajectories (latent class analysis) and defined by glycemic status as outcomes. While the primary analysis is well-described and easy to follow, these additional analyses are described mostly in the online appendix and much harder to interpret for the general reader. In my opinion, these do not add substantial information to the paper and the conclusion, and I would move these to the online appendix or drop them from the paper. I think that the paper needs a major revision however both the dataset and the study questions are adequate and important thus conditional acceptance is reasonable."*
* We'd like to thank the reviewer for this suggestion. Given that GEE models do not take into account the high dimensionality (i.e. intercorrelation) of the NEFA composition data, we ran the PLS analyses in order to confirm the GEE results when using a multivariate approach. Use of the LCMM analysis allowed us to include the longitudinal data in the PLS analysis. However, we agree with the reviewer that these analyses, while interesting in their confirmation of the GEE findings, do not contribute substantially to the overall manuscript. In light of this reviewer's comments, we decided to drop these analyses from the paper to keep the overall message of the manuscript clearer and more focused.

1. *"Title of the paper: it is a statement of the results. I would prefer to leave it open ended without an interpretation of the results, as with different levels of adjustments the results and conclusion will change substantially. See also comments on the results section."*

* As suggested, we've made the title agnostic.

1. *"Methods in the abstract: a) Please give some information on the cohort characteristics: age, sex, high risk of diabetes but no prevalent disease at baseline; b) incident diabetes cases (how were these handled? what about treated patients? - these should go in the main methods section); c) adjustment for time – I would prefer to state that time since baseline was used as the underlying time variable instead of adjusting for time"*

* We have made as many of the requested changes to the methods as the reviewer suggested without going over the word limit. Incident diabetes cases are presented for descriptive purposes though were not included in any GEE analysis. We thank the reviewer for the suggestion regarding time adjustment and agree that using time since baseline is a better variable to adjust for in the GEE models; the time variable has been replaced in GEE models, though the change does not substantially impact the final results ( … location of changes in revised MS … ).

1. *"Results in the abstract: a major problem in my view with the interpretation of the results is that all characteristics are taken as confounders although a considerable proportion of time-varying covariates are taken after the NEFA measurements. Furthermore, it is not clear whether these measures are confounders, mediators or NEFAs are mediators of these variables. I think that unadjusted results are as interesting and require discussion as the fully adjusted and selected models. Regarding the abstract, I would show both the unadjusted and the fully adjusted results here."*

* The specifics of the covariates have been expanded upon in the methods section of the revised manuscript. We have included the unadjusted results in the main manuscript and have described it in the results and discussion sections, in addition to briefly describing them in the abstract (keeping within the word limit). An explanation of our interpretation of each covariate in regard to NEFA and the outcomes (e.g. mediator or confounder) has been added in the ESM Methods.

1. *"Conclusions in abstract: I have a different interpretation of the results, particularly because adjustment waist may be an over-adjustment."*

* We have included a sensitivity analysis identifying which exact covariate attenuates the results between the unadjusted and adjusted models; the covariate is waist circumference. While inclusion of this variable attenuates most of the associations, we believe that adjustment of this variable is necessary given that biologically NEFA come from the adipose tissue during fasting and hypothetically more adipose tissue would indicate higher NEFA. Since other aspects of adipose tissue (e.g. role in inflammation and appetite regulation) could also impact insulin sensitivity and beta-cell function, we believe it is an important confounder to adjust for. We have included in the conclusions a discussion on how adjustment of waist circumference influences the model and the interpretation.

1. *"INTRODUCTION: a clear and well-written introduction with a clear objective and hypothesis"*
2. *"MATERIALS & METHODS:"*
   * *"ESM Figure 1 is not clear to me: a) Prevalent diabetes cases were excluded at baseline (n=XX); b) the lines suggest that everyone had to have a year 6 visit, ehile in the main text, the authors state that any follow-up visit was enough for being included (this corresponds well with the GEE requirements); c) what happened with incident diabetes cases? Were treated diabetes cases included in the analysis? How does that effect measures of insulin sensitivity and beta-cell function? I suggest to exclude those visits where diabetes was treated."*
   * In the revised manuscript, we have clarified how we dealt with incident diabetes cases. Briefly, we only report incident diabetes and dysglycemia cases in the text to describe the cohort, but in statistical analyses and modeling, these cases were excluded. Regarding point a) above, within the main PROMISE cohort, prevalent diabetes cases were not excluded from the study as they continued to be followed; prevalent diabetes cases did not have fatty acids measured. For point b) above,; some participants missed the 3-yr visit, but came in for the 6-yr visit. We have included wording within the Results section of the revised manuscript regarding the sample and percentage who attended at least two visits. Lastly, regarding point c) above, because diabetes can effect the values of the OGTT-derived indices due to medication the patient is taking and due to diabetes itself impacting beta-cell function and insulin resistance, we excluded them from all analyses;. To restate, diabetes cases were not included in GEE or other analyses.
   * *"I would prefer to see the equations for the outcome measures. Why not use HOMA2-IS instead of 1/HOMA-IR?"*
   * Thank you for this suggestion. We have included the equations for the outcome measures as requested. In addition, we replaced HOMA-IR with HOMA2-%S from the HOMA2 Calculator. The results were not substantially different after the replacement of HOMA-IR.
   * *"In the statistical analysis you state that co-variates were time-dependent. This is not true for age at baseline, family history, sex, ethnicity. Please clarify in the text."* ... *"misspelling: 'statistically significance'"*
   * Thanks for catching these issues. They have been corrected in the revised manuscript.
   * *"I am not an expert of PLS-DA but it is not clear to me, why does this analysis reflect more the role of relative NEFA (mole%) analysis than the concentration analysis? As I can see, it depends on what variables you feed in the model."*
   * We are not entirely clear on the reviewer's comment. PLS extracts from high dimensional data (multiple predictor variables, i.e. the fatty acids) underlying correlations, constrained by a outcome variable (i.e. beta-cell function). In this context, because mol% data is by definition restricted to sums of 100% (all fatty acid values add up to 100%), the underlying correlations between fatty acids is dependent on the relative contributions those fatty acids have to the total and as such can reflect correlations of specific fatty acids or groups of fatty acids that increase or decrease in proportion together. The concentration data is not contrained by this inherent correlation and thus underlying groups or clusters may not be identified using PLS.
3. *"RESULTS:"*
   * *"Please give ns in Table 1."* ... *"It would be also nice to see, how many participants had 1, 2 or 3 time points in the analysis."*
   * These have been added as requested. {{Tony, not sure about how I feel adding p-values to Table 1. I'm showing it purely for descriptive purposes and it might detract from discussion since, as you may recall, some variables (e.g. HDL) have a significant difference between visits, but don't really change over the visits... thoughts? I'm not too keen on generating p-values for the sake of generating p-values, esp. when it is not really relevant to the main analysis.}}
   * *"How were the changes calculated (GEE?) in the basic characteristics of the PROMISE cohort section?"*
   * The changes over time as reported in the results of the outcome variables are simple median differences between the baseline and 6-year visit. The p-value representing the difference was computed using an unadjusted (time only) GEE model.
   * *"I suggest to include the unadjusted data to the main paper (ESM Figure 3) and describe these findings as we do not know the exact model where over-adjustment start to happen."*
   * The unadjusted results and figure have been added to the main paper and described in the results section. We've also added a sensitivity analysis confirming which covariate(s) attenuates the associations.
   * *"In a trajectory analysis, the interaction between time and the main predictor (NEFA in this case) is of major interest. The authors check for this interaction only as the last step of their model building (see ESM). I would suggest to perform this as the first step of the analysis (M0), and drop this term, if it is non-significant or does not improve model fit. Furthermore, given that this interaction was dropped from the model, it is hard to except the major conclusion that NEFA predicts progression of beat-cell dysfunction. Actually what is found here is that NEFA are related to beta-cell function in repeated cross-sectional analyses and NEFA related differences in beta-cell function remain constant over time."*
   * The reviewer makes an excellent point regarding the importance of conducting an interaction check in the early stages of model building. We have incorporated this new approach in the analysis and report on the results in the revised paper. In simple terms, the reviewer is correct that without an interaction term the model is essentially "repeated cross-sectional analyses", however, GEE handles the analysis with a slightly more nuanced approach. GEE calculates the estimates of the association of NEFA with the outcomes at any given time point, taking into consideration the inherent multivariate structure of the repeated measurements of the outcome variables. As such, inherent similarities in the outcome measurements over time within a subject are included in the computation of the GEE estimates. This nuance provides substantially more power to the model and the proceeding results. See ref 27 in the manuscript for more detail on this statistical technique. Within the manuscript we have expanded on this nuance to clarify the interpretation ( … location ..).
   * *"The model building is based on literature data which is the right way to build the models but a more detailed discussion on over-adjustment is required before interpretation."*
   * To clarify, the model building processes used two additional techniques: quasi-likelihood information criteria (QIC) and directed acyclic graphs. The results and output of these techniques can be found in the ESM. Combining all of these processes in building the model ensures that adjustment is empirically based and reduces (but not eliminates) the potential for over-adjustment. However, we have added additional sensitivity analyses to identify covariates that strongly influence the results and discuss the results in light of these findings.
   * *"Latent classes. Although this is a very attractive method, based on ESM figure 4, there are 3 parallel declining group trajectories that is mostly affected by the baseline values. Groups based on baseline (last follow-up) tertiles would be easier to interpret."* ... *"The PLS-DA analysis needs further description. I would expect variables with the highest loadings to be the variables in the unadjusted GEE analysis. I am probably wrong given the results."* ... *"Do we really expect good discrimination? The associations are week to nul between NEFAs and the outcomes. I do not really see the point the extensive supplementary analysis on PLS-DA. Furthermore dysglycemia is an outcome that is not directly related to beta-cell function only. Dysglycemia requires both insulin resistance and beta-cell dysfunction."*
   * Given this reviewer's previous comments and suggestions, we have decided to: focus on the GEE analysis in the main paper; remove the LCMM analysis; perform the PLS analysis on the baseline (cross-sectional) data using the outcomes as continuous variables; and, briefly describe the findings in the Results section. To keep the ESM material streamlined, we have contained the PLS results and other additional analyses conducted within the code (accessible via the DOI) for the interested reader. We believe this simplifies the results and primary findings of this paper without sacrificing a loss in the potential use these additional analyses may provide for the interested researcher.
   * *"The authors mention in the Discussion the external validity is mostly limited to white females. I suggest to run a sensitivity analysis on this group."*
   * We agree with the reviewer's comment and ran stratified models by either sex or ethnicity. In both cases, all significant associations from the non-stratified analyses were attenuated, likely due to the reduction in sample size in the stratified models. Stratifying analyses has the limitation of substantially reducing sample size and is the reason why interaction testing is prefered. We ran interaction tests by sex and ethnicity and found no statistically significant interactions. While these results internally suggest no differences between sex or ethnicity, there may still be features unique to sex and ethnicity in this cohort that limit generalizability.
4. *"DISCUSSION"*
   * *"I would like to see more on the DAGs that underlies the selected variables in the models. That would either confirm the way the analysis is currently performed, or would show that some of the adjustments are over-adjustment."*
   * As requested, we have included the DAGs underlying the hypothesized associations in the ESM.
   * *"In the time-varying analysis some covariates are measured after NEFAs, thus adjustment for them could be over-adjustment just because they are measured closer to the outcome of interest."*
   * In order to confirm the impact the comment made by the reviewer has on the reported associations, we ran sensitivity analyses where we had all covariates as time-independent, to match the NEFA variables in the GEE model. We found that there were negligible differences in the results between models with covariates as either time-dependent and time-independent and models with all covariates as time-independent.

# Associate Editor

* *"Many thanks for submitting this potentially interesting paper. The second reviewer comes with some very helpful comments that will need to be addressed in detail. There is need to consider the results from the Paris Prospective study: Charles MA et al. The role of non-esterified fatty acids in the deterioration of glucose tolerance in Caucasian subjects: results of the Paris Prospective Study. Diabetologia 1997;40:1101-6"*
* We thank the associate editor for the kind comments. We have included a discussion of the cited paper in the revisedDiscussion section.