Author's response to reviews

Title: Identification of a prognostic signature for old-age mortality by integrating genome-wide transcriptomic data with the conventional predictors: the Vitality 90+ Study

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Author's response to reviews: see over
Covering letter

Dear Professor Frank R Sharp,

We are grateful for the opportunity to revise our manuscript “Identification of a prognostic signature for old-age mortality by integrating genome-wide transcriptomic data with the conventional predictors: the Vitality 90+ Study” (MS: 5386578761325159).

Below, please find point-by-point answers to the Reviewers’ comments

All authors have red and approved the manuscript and the manuscript has not been, or is not, under consideration for publication in another journal, in whole or in part, in any language. The study protocol has been approved by the local ethics committee and an informed consent was obtained from all of the participants.

Sincerely,

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Authors’ responses to the Reviewers’ comments

We would like to thank the Reviewers for their valued comments and suggestions. We have now addressed each of the comments and modified the manuscript accordingly. Below, please see the point-by-point answers to the comments. All of the changes made to the text have been underlined. The manuscript has also been edited for English language by the American Journal Experts-service (certificate available from the authors upon request). Thus, some (minor) changes have also been made to the text due to the language editing.

Reviewer's report

Title: Identification of a prognostic signature for old-age mortality by integrating genome-wide transcriptomic data with the conventional predictors: the Vitality 90+ Study
Reviewer: Glen Jickling
Reviewer’s report:
Survival models of demographic markers were compared to gene expression markers in 151 nonagenarians. RNA was isolated from PBMC by Ficcoll-aque stored in RNA later, and processed on Illumina BeadChips. 478 transcripts were identified (p<0.05), of which 378 were associated with mortality after adjusting for BMI, frailty index and cell free DNA.
1. The statistical selection criteria for genes associated with mortality could be more clearly presented. A p<0.05 appears to have been used, which does not adjust for multiple comparisons. The strength of the association of each of the 478 transcripts identified with mortality is unclear. Was a fold change between those who died and did not die applied?

Authors’ reply: We acknowledge that the selection criterion (p<0.05) for the mortality-associated genes was indeed poorly presented. We have now added a sentence to the Methods-section clarifying the selection criteria for these transcripts (page 9, lines 7-8). The strength of the association between each of the 478 transcripts and mortality in the univariate Cox model was presented in the original submission, as well as in this revision, in an Excel file named “Additional Table A1” in which the strength and directionality of association for each transcript is represented by its respective z-score, p-value and HR. We hope that sufficiently explains the comment concerning the “strength of association”. Please see below for the answer to the comment regarding the use of fold change.

Because we chose to use penalized regression modeling to obtain the final mortality signature from the 331 transcripts that remained as independent predictors after adjusting for BMI, frailty index and cf-DNA level, we followed the guidelines introduced in pioneering papers implementing penalized regression models into survival prediction using whole-genome transcriptomic data (Bøvelstad et al. Bioinformatics. 2007 Aug 15;23(16):2080-7; Bøvelstad et al., BMC Bioinformatics. 2009 Dec 13;10:413.); thus, we did not perform correction for multiple testing. This protocol did not include correction for multiple testing because the penalty term lambda essentially performs a similar task. Briefly, the penalized regression models rank the variables according to the extent that each of them alone explains the variation in the outcome (mortality rate) and excludes (imposes more penalty) those variables that are less relevant to the outcome, that are collinear and/or that would produce overfitted models. Although this operation is basically independent of the traditional p-values and z-scores obtained in the Cox multivariate pre-selection analysis (Additional Table A2), all of the 16 top-ranking transcripts identified by the Ridge regression model (Additional Table A4) were among the top 50% significant (p<0.025) genes identified in the Cox multivariate model (Additional Table A2). We have now added a few lines explaining the methodology (Methods-section, page 9, lines 12-15).

In this study, we did not use any two-group comparison tests, such as the empirical Bayes (or an equivalent), which would have produced fold changes to represent the differences in gene expression levels between the compared groups. Instead, we only applied the univariate and multivariate Cox regression models and the penalized regression models, which are not pure group comparison tests, because survival time was also taken into account in these models. We considered models including time-dependent covariates to be the most appropriate way assessing mortality predictors in very old individuals because the groups (survivors vs. non-survivors) are not so drastically different from each other that all the relevant information would be obtained from the models without including the survival time. This situation would indeed
differ in case-control settings and acute clinical settings.

2. Prediction analysis was performed on the derivation cohort using cross-validation. A second validation cohort would be a better method to evaluate the gene predictors of mortality.

Authors’ reply: We absolutely agree with the Reviewer that a separate validation cohort would be the best way to assess the predictive performances of the gene predictors. However, to the best of our knowledge, corresponding general population-based nonagenarian cohorts in which both the mortality follow-up data and a whole genome transcriptomic analysis from peripheral blood mononuclear cells are available do not exist at the moment. We do recognize that the lack of a second cohort is a major limitation of our study, and we have stated this in the manuscript (Discussion-section, paragraph starting from the bottom of page 18).

3. It would be useful to present table 1 divided by those who died vs those who survived. Currently it is difficulty to determine which factors are different between the compared groups.

Authors’ reply: We have now divided Table 1, as suggested, and have presented the variables separately for those who did and did not survive. However, we did not include the statistical significances for the between-group (i.e., survived vs. died) differences in the variables to avoid confusion with the mortality-associated variables identified using the Cox regression model (presented in Table 2).

4. The number of study subjects that died should be more clearly presented. Cause of death would also be of interest. Might different inflammatory genes be associated with specific causes of death?

Authors’ reply: We regret the unclear presentation of the number of subjects who died during the follow-up and have now stated this more clearly in the Methods section (page 5, lines 12-14.) We have also added this information to the Abstract (page 2, 4th line in the Methods-paragraph).

We agree with the reviewer that addressing the mortality-predicting genes according to the cause of death would be of utmost interest and importance. However, at this point, when only 49 individuals died, the models would not have sufficient statistical power and accuracy to identify the cause-specific predictors, and the results would remain speculative. Nevertheless, we are continuing to collect mortality data from this cohort periodically and will most certainly analyze the cause-specific mortality signatures once all or at least the vast majority of the individuals have died. We thank the Reviewer for pointing this out and regret that we are currently unable to address this issue.
Reviewer's report
Title: Identification of a prognostic signature for old-age mortality by integrating genome-wide transcriptomic data with the conventional predictors: the Vitality 90+ Study
Reviewer: Natalia Gavrilova
Reviewer's report:
This is an interesting paper intending to find predictors of old-age mortality. However some issues in this paper raise questions.

It is not clear whether the authors included sex variable in their analyses. If they included and sex turned out to be non-significant predictor of mortality, then authors should clearly state this in their paper and explain why this strong mortality predictor does not work in their sample.
Authors’ reply: Yes, we initially did test the effect of sex on mortality in the Cox univariate model, and it was found to be non-significant (p=0.476). We have now stated this issue in the Results section (page 12, lines 1-3) and provided a probable explanation for the lack of association in the Additional methods -file (the first paragraph on page 3). We suggest that the most likely explanation is that the men in this cohort were in better condition on average, and possessed better functional capabilities compared with the women, and this phenomenon compensated for the generally higher risk of mortality observed in elderly men. Specifically, there was a smaller proportion of frail individuals among the men than among the women (31.58% of men vs. 47.92% of women), and the opposite was true in the non-frail category; there was a greater proportion of non-frail individuals among the men than among the women (68.42 % of men vs. 52.08 % of women).
Similarly, the men of this cohort had higher median Barthel index scores (93.56 for men vs. 90.52 for women) and MMSE test scores (25.20 for men vs. 23.66 for women). Because being non-frail and having higher MMSE and Barthel scores were protective against mortality in our study (Table 2), we assume that these factors largely explained the fact that male sex was notobserved to be a statistical risk factor of mortality. These analyses and results are presented in detail in the Additional methods -file (the first paragraph on page 3). We thank the Reviewer for pointing this important issue out.

Also, at this high age even 6-month difference in age may have profound effect on mortality, so that age expressed in months should be included in the analyses.

Authors’ reply: We have now analyzed the effect of age in months on mortality, which was not significant (p=0.654). We have now also added this information, i.e., age in months to Table 1 and stated this result in the Results section (page 12, lines 3-5).
Using continuous scale for such variable as BMI, which have U-shaped dependence on mortality may be inappropriate. The same may be true for other continuous variables.

Authors’ reply: We thank the reviewer for pointing out this important issue. We assessed whether the relationship between BMI and mortality deviated from linearity. We observed that in our cohort, this relationship followed linearity and there was no evidence of a U-shaped relationship. We assessed this by simultaneously adding both a linear BMI and a 3-class BMI (i.e., the variable converted into tertiles) to the Cos regression model. If the relationship had been non-linear (e.g. U-shaped), then both the linear and 3-class BMI would have remained in the model. However, only the linear variable remained as a predictor as indicated below:

```
.stcox bmi
No. of subjects =          151                     Number of obs   =       151
No. of failures =           49
Time at risk    =  334.9150685
Log likelihood  =   -231.12316                     LR chi2(1)      =      8.97
                       Prob > chi2     =    0.0027
------------------------------------------------------------------------------
   _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
  linear BMI |   .9033542   .0320102    -2.87   0.004     .8427444    .9683231
------------------------------------------------------------------------------
```

```
.xi: stcox bmi i.3-class BMI
No. of subjects =          151                     Number of obs   =       151
No. of failures =           49
Time at risk    =  334.9150685
Log likelihood  =   -229.86976                     LR chi2(3)      =     11.48
                       Prob > chi2     =    0.0094
----------------------------------------------------------------------------------
   _t     | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+--------------------------------------------------------------------
  linear BMI |   .8060719   .0644353    -2.70   0.007     .6891772    .9427937
  3-classBMI(2.cl)|   2.168045   1.134378     1.48   0.139     .7774951    6.045595
  3-classBMI(3.cl)|   4.515346   4.518241     1.51   0.132     .6352486    32.09507
----------------------------------------------------------------------------------
```

These results have now been added to the Additional methods -file (page 3, second paragraph).

Further inspection of this matter confirmed the result: of those (n=49) who died during the follow-up, 25 individuals (51%) had BMI values <25, whereas only 6 (12.2 %) had BMI values ≥30. In addition, a T-test comparing of the BMI values of the survivors and non-survivors demonstrated that the non-survivors had significantly lower BMI values than the survivors (24.8 vs. 27.1; p=0.006).
Similar tests were performed for waist circumference, hip circumference and systolic and diastolic blood pressures, none of which displayed non-linear relationships with mortality (Stata output files for these results are available from the authors upon request).

The paper uses very small study sample and their models are overloaded with variables. This should be mentioned as limitation. Generally, the limitations are not clearly stated.

Authors’ reply: We agree with the Reviewer that the small study sample is an obvious limitation of our study. We have now added a paragraph stating the limitations of the study (Discussion-section, paragraph starting from the bottom of page 18). However, the “overloaded” models, which included multiple variables, were performed using penalized regression modeling (Ridge regression) which is specifically designed for situations in which the number of variables largely exceeds the number of individuals (for details of the model descriptions, please see Bøvelstad et al. Bioinformatics. 2007 Aug 15;23(16), and Verweij and Van Houwelingen. Stat Med 1994, 13:2427-2436). The Ridge regression, as well as the other tested models, i.e., the Lasso and C-index boosting algorithm, are widely used nowadays to assess high-dimensional (and typically multicollinear) whole-genomic data. These models are able to pick up the most significant predictors even among thousands of variables. By applying this methodology, in the subsequent traditional Cox regression model (Table 4 and Additional Table A3), we were able to follow the general guideline of including 5-10 events per predictor variable.

In addition to genome-wide expression studies, penalized regression models are also used in genome-wide methylation and association (GWA) studies, in which the number of modeled variables is always much greater than the number of individuals. Please see for example:


Finally, the structure of article is not convenient for reading because the Methods section is placed in the very end of the article.

Authors’ reply: We regret our previous placement of the Methods-section was placed at the end of the article, and we have now moved it immediately following the Background-section.