Author’s response to reviews

Title: Annual decline in forced expiratory volume and airway inflammatory cells and mediators in a general population-based sample

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Cover Letter

We appreciate the efforts of the Editor and Reviewers to evaluate and comment on our manuscript, which we believe has improved the manuscript.

Editor Comments (EC):

EC1: “Notably, the quality of written English was quite poor. We highly recommend including a co-author who is a primary English speaker to help with language.”

Authors’ reply: We regret that the quality of our English language was poor. Now, the manuscript has been edited by to San Francisco Edit according to the BMC Pulmonary Medicine guidelines. Thereafter we have evaluated their revisions regarding the content and added the changes that have been suggested by Editor and the reviewers.

EC2: “Additionally, there were concerns about the methodology including lack of adjustment for confounding factors, and very small sample size for some comparisons. A thorough revision
including such edits would be necessary in order for this manuscript to be considered for publication.”

Authors’ reply: Regarding inclusion of confounding factors, see our reply to WL1. Likewise, we have removed the last column in table 4, and changed the text in the abstract and the result chapter, see our reply to OH17. We have done our best to respond to reviewers’ comments.

Olaf Holz (Reviewer 1), (OH):

OH General:

OH1: “Olaf Holz (Reviewer 1): Please include all comments for the authors in this box rather than uploading your report as an attachment. Please only upload as attachments annotated versions of manuscripts, graphs, supporting materials or other aspects of your report which cannot be included in a text format.

Please overwrite this text when adding your comments to the authors.”

Authors’ reply: In submission of the revised manuscript and our comments to the reviewers, we are following the submission guidelines made by the Journal.

OH Major:

OH2: “1. The ratio for this study could be better described. I miss potential epidemiological data. There are studies from Canada and Italy available about normal sputum composition. Especially sputum neutrophils seem to differ between regions. Is there evidence, that e.g. environmental factors like pollution actually lead to different declines in lung function between differently polluted areas. In our experience (unpublished) weather and temperature (potentially linked to an increased exposure to pathogens) also seems to affect neutrophil levels in sputum. There is published data on the effect of aging on sputum composition (ref 18).”

Authors’ reply: OH is one of the best experts in this field, and we agree that region is an important point here that should be added to the manuscript. In the revised version we have added new text to include OH1’s comment on this point, see page 5, lines 5-10 and lines 14-18.

OH3: “1. continued… In this respect it seemed a bit surprising, that the authors selected a normal population sample, which was actually quite old … mean age 55 at baseline. Why not younger?”
Authors’ reply: There are two reasons for selecting participant aged 40 years or older:

1. They were invited to participate as a reference group in a study of sputum composition among patients with myocardial infarction in 2003 – 04.

2. The prevalence of accelerated decline in FEV1 occurs from approximately from the age of 40 or older (ref. 14).

OH4: “2. The authors should potentially revise the arguments provided on page 16 in paragraph 2 and 3 (line 6, 14). The interindividual variability of induced sputum cell composition was well known in 2003. The same is true for the approximate decline in lung function in the general population. Therefore it does not seem to be appropriate to argue after the end of the trial, that the study was potentially underpowered.”

Authors’ reply: Taken into account, page 16, para 2 and 3 removed from the manuscript.

OH Minor:

OH5 “1. Not being a native English speaker, I still believe that the language of the manuscript could in parts be improved.”

Authors’ reply: Already taken into account, see answer to Editor (EC1).

OH6 “2. Is there a major reason for the rather large number of subjects that declined to participate (n=44)?“

Authors’ reply: According to Regional Ethical Committee’s approval, we were unable to ask why the participants would not meet to the follow-up.

OH7 “3. "All the participants were offered a reversibility test" … how many did accept this?”

Authors’ reply: 56 participants accepted a reversibility test. Added in to the text page 8 line1, 2.

OH8 “4. The description of sputum induction is not clear. All participants inhaled first 0.9% and all participants then inhaled 3, 4, and 5%? Each inhalation period lasted for 7 minutes? The statement "until a sufficient amount was delivered" suggests that some subject only e.g. inhaled for 5 min others e.g. for 20 min. The danger in doing so is, that it could affect sputum
composition, as the first portions produced are generally rich in neutrophils and later portions richer in macrophages.”

Author’s reply: Duration of inhalation was different in different participants and we used the whole expectorate excluding saliva prior. However, we have not noted how much NaCl concentration each patient needed.

OH9”5. Was the sputum selected from saliva prior to processing? Or was the whole exspectorate processed. This is important to interprete the biomarker levels in the supernatant. “Author’s reply: Sputum was selected from the prior saliva. We have removed the excess sputum from the sample.

OH10”6. Cytospins were evaluated and not smears, as stated in page 8 line 12.”

Author’s reply: We counted cells from cytospins not from smears.

OH11 ”7. The provided additional file shows the agreement between 2 evaluators. From the text page 8 I thought this refered to the differential cell count, but the axis in the figure appears to refer to cell numbers/ml sputum and it is not clear for which cell type. Please clarify.”

Author’s reply: It was originally planned to use cell/ml but we changed our minds to use percentage distribution and it became many plots. We have decided to remove this additional file in the revised manuscript.

OH12”8. Table 1: what does Packyears refer to… the mean of all smokers (active and quitters?)”

Author’s reply: Yes, it refers to the mean pack-years of all the participants. Pack/years were calculated by multiplying the number of packs of cigarettes smoked per day by the number of years the person has smoked. It is added to the text. Added to the text page 8, line 1,2.

OH13. ”9. Table 2. Tertiles numbers do not add up to 62.”

Author’s reply: There was a typo in the submitted manuscript – it is corrected in the revised version.

OH 14”10.dFEV1 Table 2: 33.0 in abstract 32.9… should be consistent.”
Author's reply: It is changed from 33.0 to 32.9 in table 2.

OH15.”11. How many started smoking during the observation period? Page 12 top paragraph.”
Author's reply: No one started to smoke during the observation period according self-administered questionnaire. Added to the text, page 11, line 5.

OH16.”12. Table 3: the levels of sputum cytokine appear to differ from the literature, which is likely to the use of the bead multiplex assay. IL8 levels seem to be rather low, and very similar to IL6 levels, which are generally lower in sputum. IL17 subtypes on the other hand appear to be very high. In such a large sample size there should be a correlation between log IL8 levels and log number of sputum neutrophils. Did the authors see such a correlation? “
Author's reply: Yes, it is highly significant correlation between logIL8 and log number of sputum neutrophils (Pearson correlation). This is added into the text, see page 14, line 8,9.

OH17.”13. Table 4: Comparing 5 vs 49 subjects for me is already borderline, comparing 2 vs. 49 does not seem to be appropriate. The data about these subgroups should be mentioned and discussed, however, at least for ACO the statistics should be removed.
Author's reply: This column is now removed from table 4, and the text in the abstract (page 3, lines 16 – 19) and result chapter (page 17, line 3) is changed accordingly as there were some details here that we believe is important. Moreover, we made some changes to the first paragraph of the discussion, page 17, lines 20-22.

OH18.”14. I do not think that a small sample size was a limitation of the study. 62 subjects is quite a reasonable number. With this sample size it should be possible to detect a clinically relevant relationship between airway inflammation and the decline in lung function.”
Author's reply:
We thank the professor Holtz for this comment, nonetheless, we still have some concerns (page 19, lines 18 – 21).
WL1: "The reported mean decline in FEV1 was 32.9 ml/year for the cohort. This decline could be mostly due to the effect of advancing age. If we consider a Caucasian man with the mean age reported in the study (55 years) and the mean height reported in the study (1.74 m), his expected FEV1 is 3.58L based on the NHANES spirometry reference equations. The expected FEV1 of the same man 10 years later (i.e. at age 65) is 3.25L. His decline in FEV1 over 10 years is 3.58 - 3.25 = 0.33L = 330 mL which equates to a decline of 33 mL per year, the same mean decline observed in the cohort. This overall relatively small decline in FEV1 is not surprising as the recruited participants were reportedly healthy at baseline with a limited smoking history. Along the same lines, categorizing the decline in FEV1 by tertiles may not be physiologically accurate. If we look at the highest tertile (> 38 mL/year), such a decline may be expected for a tall man but may be indicative of disease in a short woman. Therefore, given the aforementioned observations, to more accurately assess the effect of inflammatory markers on FEV1 decline, it may be better to analyze it while controlling for other factors that can influence spirometry results such as age, sex, height and smoking history (e.g. in a multivariable model)."

Authors’ reply: We investigated the association between neutrophils and dFEV1 in a linear regression model using as age, sex, height and smoking history (expressed as pack-years) as covariates. It turned out that the percentage of neutrophils increased significantly with increasing number of pack-years (0.444%/pack-year, p = 0.015), whereas the other coefficients in this model were non-significant. We have not made any changes to the text because we still have some concerns (page 19, lines 18 – 21).

Additional comments WL:

WL2: “Although a positive reversibility test is a common feature of ACO, it may not be enough alone to diagnose it.

- On page 9, lines 8-9, the authors state that "there were no significant differences between total, follow-up and not follow-up groups." I recommend just directly comparing the characteristics of the follow-up and not-follow-up groups (while still showing the total in the table).”

Authors’ reply: This is exactly what we have done. There is a typo in the text. It is corrected in the revised version of the manuscript, see page 11, lines 1,2. (The language is also change as suggested by the language editor).
WL3. “For comparisons in Table 2, unless the authors are working with normally distributed data, I recommend using a Kruskal-Wallis test instead of a one-way ANOVA test to compare characteristics across the three groups given the small number of subjects in each group.”

Author’s reply: Only blood cells were not normally distributed in Table 2 and we used log transformed data and one-way Anova test (parametric test). We also used the Kruskal-Wallis (nonparametric test) but the results were unchanged.

WL4. “There seems to be a number of discrepancies in the raw data shown in Table 2 of which I will point out a few: a) the number of participants in the groups (20, 22 and 19) add up to 61 (not to the 62 subjects with follow-up data)

b) the number of reported males is 61 (should be 35?), c) the mean monocyte count for the total group is reported as 0, which is unlikely given that two of the tertiles have mean monocyte counts of 0.4 each.”

Author’s reply: We thank the reviewer for these comments.

a. The number of participants in the group was 19, 20 and 23.

b. The number of reported male is 35.

c. The mean monocyte count for the total group is 0.4.

These typos are now corrected.

WL5. “In Table 4, only two subjects have post-BD FEV1/FVC ratio < 0.7 with reversibility, which makes it hard to draw any definitive conclusions regarding this subgroup.”

Author’s reply: it is removed from the table 4 and discussed in the text, see out comments to OH17.

WL6. “Furthermore, in Table 4, given the low number of subjects being compared in each group, a non-parametric test such as Mann-Whitney test would be recommended instead of a two-sample student t-test.”

Author’s reply: Table 4 is now changed, and the number of participants in the revised version is now seven or 49. We believe that this number is ok for Student t-test.
WL7.”Again, there seems to be a number of discrepancies in the raw data in Table 4 of which I will point out a few: a) the number of participants in the groups (49, 5 and 2) add up to 56 (not to the 62 subjects with follow-up data),”

Author’s reply: The number 56 is correct because only 56 subjects completed reversibility test and 7 of them had Post-BD FEV\textsubscript{1}/FVC-ratio < 70 %, see also our reply to OH7.

WL8. “b) the percentages of females between parentheses (on the last line of the table) may be incorrect.”

Author’s reply: These percentages are now corrected in the table 4. In addition “female” replaced by “male” because male is used as the index for sex in tables 1 and 2.

Christine Freeman (Reviewer 3),(CF):

CF1.” In the Introduction, page 4, line 22, the authors note that subjects who developed COPD had accelerated dFEV\textsubscript{1}. However, recent data suggests that COPD can also be the result of failure to achieve normal lung function followed by a normal rate of decline. This is discussed in a recent perspective piece (Martinez FJ, et al., At the Root: Defining and Halting Progression of Early Chronic Obstructive Pulmonary Disease. 2018 Am J Respir Crit Care Med PMID: 29406779). The authors might want to address some of this newer data in their introduction or discussion.”

Author’s reply: We thank the reviewer for this comment. It is added in to introduction page 6, line 12-14.

CF2.”Did the authors look at the respiratory symptom questionnaires to determine whether sputum markers correlated with any symptom-related scores?”

Author’s reply: No, we did not. It might be a subject of separate investigation.

CF3.” On page 7, line 20, the authors state that visible squamous epithelial cells (SQE) were excluded from the total cell counts. High numbers of SQE contamination could have an impact on the sputum sample. Did the authors use all samples, regardless of SQE numbers, or was there criteria established to determine if a sample should be rejected? If all samples were used, SQE numbers should be reported.”
Author’s reply: The sample was rejected if SQE contamination was 20% or more and we had to exclude some sputum samples. Added to the text page 9, line 12, 13.

CF4. "What criteria were used to determine current vs former smokers?"

Author’s reply: Former smokers had to be quitters for at least one year or more. Text added to the revised manuscript page 7, lines 21 and page 8 line 1, 2.

In addition – erratum in the submitted manuscript: In paragraph 1 of the discussion “no” was lacking: it is now added in to para 1 page 17, line 16.