Author's response to reviews

Title: Elevation of Sputum Matrix Metalloproteinase-9 Persists up to 6 Months after Smoking Cessation

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Author's response to reviews: see over
Answers to reviewers comments

Reviewer: Simon Johnson
Reviewer's report:

Major Compulsory Revisions
1. I assume that all people completing the study had measurements at baseline and 3 and if still abstinent 6 months but this isn’t clearly stated.

It is true that this was not informed clearly, a sentence about this is now added to the results section.

2. The number of patients/controls per group should be stated in figure 1 to make it easier to assess how robust the data is.

This is a good comment. The Figure legend has been corrected accordingly

3. The table gives clinical details of all patients, the ones of interest are only those that completed the study and this should be removed unless they also contributed to the baseline values. If so this should be made clear.

This is again an important concern. We have now included another Table (Table 2), which contains the subject data of those who had quitted for 3 months and those who quitted at least for 6 months.

4. The difference between the temporal profiles between MMP-7, -8 and -9 is interesting if this temporal profile is actually different between the MMPs. Is the fold change significantly different for the different MMPs across the time points? Not just compared with themselves at baseline.

It might be that we did not understand this question completely. Anyway, we have compared each MMP separately to its level at the baseline i.e. we can conclude not only how the different MMPs change in induced sputum of smokers with or without mild asthma or COPD but also how each MMP behaves after smoking cessation compared to the baseline of that specific MMP. This has been clarified in the revised manuscript.

Minor Essential Revisions
1. If MMP-9 is persistently raised this may be in keeping with the findings of Lowrey et al who showed that MMP activity was lower in patients with more severe COPD compared with healthy smokers, this could be discussed.

This is true. This reference is now added to the discussion section.
Reviewer: Brigita Sitkauskiene
Reviewer's report:

Major comments:

1) As the authors describe, smoking cessation is an important factor for the reduction of COPD and other airway inflammatory disorders progression. Twenty five asymptomatic smokers, 15 smokers with asthma, and 21 with chronic bronchitis and COPD were recruited to the study (according to the Table 1).

The methodology/design employed in this study may be appropriate; however, the results obtained from all these groups were analyzed as pooled group. That is why it is unclear whether high levels of MMP-9 in induced sputum are present in COPD patients or other groups before and after smoking cessation (as well as levels of neutrophils, MMP-7, -8, TIMP-1).

We understand that this was unclear. We added Table 2 to clarify this topic, where only those who quitted smoking have been included. There is overlap in smokers with symptoms, mild COPD and asthma. Since the number of the individuals in single groups is unfortunately too low for statistical evaluation (no statistical power), the groups cannot be evaluated separately. However, we can make important conclusions how various MMPs change after smoking cessation (key finding of this study).

Moreover, it is known that expression of MMPs may be induced not only by smoking, but by the disease itself (Demedts et al. Thorax 2006; Babusyte et al. Respir Res 2007). Analysis of the results from all groups (‘healthy’ smokers, patients with asthma and COPD) should be presented and discussed separately. If these groups are too small for the statistical analysis, the study should be extended.

This topic is important, and it is included with references in the Introduction and Discussion sections that the disease itself might induce MMP expression.

2) Furthermore, analysis of relation between (possible) decline of lung function and studied inflammatory markers, smoking history (pack-yrs) could be a matter of scientific interest.

This is impossible to evaluate since it is known that most benefits for smoking cessation e.g changes in lung function are measurable after several years, not in months.

Minor comments:

1) Introduction is a bit too long, and should be more addressed to the topic.

We have shorted Introduction by deleting a couple of sentences. We, however, feel that most of the background presented in Introduction is needed to better understand the complexity (what we know and what we do not know) for the evaluation of smoking cessation based on induced sputum specimens
2) It is incorrect to call the smokers without respiratory symptoms as healthy (asymptomatic inflammation may persist).

Yes, we agree. This is now corrected to “asymptomatic smokers “ in Table 1.

3) Asthma was defined according to GINA? (Ref.?)

Yes, asthma was defined according to GINA and this reference is now added to the methods section.

4) The subjects after smoking cessation were provided with ‘available pharmaceutical therapies’. They should be indicated as well as producers.

Pharmaceutical therapy was offered for each individual and was based on the doctors’ assessment. Varenicline was not registered in Finland during the study.

5) In Methods the authors indicate that some of patients showed positive skin test; however, they do not analyze or discuss this fact in the manuscript.

It is true that this was inadequately explained. The listed patients showed positive reactions in the skin prick test analyses for some of the common aeroallergens (birch, grass, mugwort, Cladosporium herbarum, cat, dog, horse, and/or the house dust mite Dermatophagoides pteronyssinus). This has now been explained more detailed.

6) “Tryptan blue” must be “Trypan blue”

This was spelled incorrectly but is now corrected as “Trypan”.

7) Cytocentrifuge preparations were made by Cytospin (manufacturer?).

The Cytospin used was Shandon Cytospin 3 Cytocentrifuge and is now added to the text.

8) In results: “The inflammatory profile in the sputum revealed neutrophil predominance as is often the case also in smoking asthmatics “ The sentence is a part of Discussion.

This sentence is now modified.

9) Results, last sentence: “… the levels remained elevated (p#0.069)”. According to the ‘p’, result is non significant, that is why the proposition is incorrect.

This was a bit unclearly explained but what we meant was that after 6 months of smoking cessation, the sputum MMP-9 levels did not change significantly when compared to the baseline, so the ‘p’ is really non significant. This is now explained more clearly.
Reviewer: Charles McSharry
Reviewer's report:

The authors could comment on the significance of MMP7, 8 and the clinical significance of their reduction.

This is true. We have added a few sentences in the Discussion section about this important topic.

Was there any correlation between lung function and any sputum biomarker?

Correlations were not calculated the reason being the low number of individuals, the statistical power remains weak, or the correlation (if received) may even give incorrect conclusion.

Was there any correlation between neutrophil proportion or absolute count and MMP9?

This would have been interesting but because of the low number of individuals correlations were not calculated. Please see also the answer above.

The longitudinal data should be shown as individual dot plots and analysed accordingly to show if the changes were consistent between individuals and not random between individuals.

This is very important point, the changes in the individual values have now been shown in the revised Figure 1. The levels of the neutrophils, MMPs and TIMP-1 in non-smokers are now included in the text (otherwise there are too many Figures for this short paper.

The largely normal lung function at baseline suggests that these were not very compromised patients. This study would be more informative using patients that would allow biomarker comparisons to be made with any improvements in lung function. It is difficult to interpret changes in MMPs if the lung function changes are unknown therefore the last sentence in the abstract referring to ongoing lung damage in the conclusion is unwarranted.

This is a good point. However, 6 months is a too short period to evaluate changes in lung function since it is known that possible changes in lung function after smoking cessation are detectable after several years. The present study was planned to evaluate if specific markers that have been associated with the pathogenesis of smoking related airway diseases can give even more information how the remodeling/injury mechanisms in the airways will normalize (more sensitive than spirometry) after smoking cessation.

The striking observation of a high MMP9 at 3 months probably deserves more critical assessment in the discussion.

This is important comment, and we have included this in the Discussion.
Reviewer: George Leikauf

Reviewer's report:
Major Concerns:
1. Is the question posed by the authors well defined? No.
The scientific question addressed in this study was compromised by the high drop out rates of the subject who attempted to stop smoking. In the introduction, the authors attempt to address the similarities and difference between inflammatory cell types and proteinases/antiproteinases in lung of persons with asthma and COPD. This background seems to suggest that there might be difference in these subgroups in the level of inflammation and proteinase that would be altered during smoke cessation. However, there is not enough members of each group (non-asthmatic/non-COPD, asthmatic, mild COPD and moderately severe COPD) at the end of the study. All the subject groups are combined for the analysis. Therefore, the question posed is “if smoking cessation is associated with changes in numbers of neutrophils, MMP7, MMP8 , MMP9 and TIMP1 levels in induced sputum specimens” in persons with and without asthma or COPD. The attempt to obtain data from different clinical subtypes diminishes the power of the study greatly. Thus, the study can only be viewed as preliminary. A case crossover design using only asymptomatic smokers would be a useful improvement. Unfortunately, based on dropout rates, the study probably would need to recruit about 300 individuals to obtain data on 45 individuals at 6 months. Each subject will serve as their own control, however.

This is true. We started a large study, which was partly disappointing since so many smokers did not stop smoking (which actually is true based on many other studies). The high number of dropouts caused that the number in individuals who succeeded in quitting remained small and thus diminished the power of this study. We agree, that our results can be considered only preliminary and further studies are needed to confirm the results. This may, however, be the reason why corresponding studies have not yet been published. This study anyway gives some information suggesting that various MMPSs (which have been suggested to associate with airway pathology) do not immediately normalize after smoking cessation and their decline may differ. Moreover, especially the function of various MMPs may differ which should be investigated more detail before final conclusions from this or other studies can be obtained. These factors have been added to the Discussion section.

2. Are the methods appropriate and well described? No.
The statistical analysis is inappropriate. It is difficult to determine what the mean and standard deviations were for each group. Multiple comparisons are being done, which requires ANOVA with correction. The main comparison of interest in Figure is the smoker 6 month values compared to the smoker baseline. Both MMP7 and MMP8 seem to have improved and it difficult to determine why these value at 6 months are not statistically different from the baseline values for this group. Also it is not clear what the sample size is for each group and this should be added to the figure legend (is it 30 nonsmokers vs. 17 smokers at each time after cessation?)
We agree that this remained unclear. The n numbers have now been added to the Figure legend. We also added Table 2 about the clinical characteristics of those subjects who completed the study. Moreover, Figure 1 has been changed to show the profile of each MMP in each individual, the statistics have been clarified, it was based on the values of each individual compared to his/her value at the baseline.

3. Are the data sound? Yes, but only preliminary. The sample size is small and statistical methods are of concern. Also the method of measurement of MMP does not include a measure of the enzyme activity, but only the amount of the enzyme present. Activity could and should be measured by zymography or other commercial methods.

It is true that the ELISA system does not measure activity. However, ELISA system is also widely used in previous studies. Moreover in many corresponding studies on sputum MMPs (also in our recent study) MMP-ELISA levels have been found to correlate with the activity at least in case of MMP9 (gelatinase). A few sentences about this have been added to the Discussion section.

4. Does the manuscript adhere to the relevant standards for reporting and data deposition? No. Table 1 provides the data on the starting population, but Figure 1 is only a subset of the recruited population. The Subject characteristics for the remaining population should be report systematically.

We agree about this. Table 2 is now added about those 17 subjects who completed the study.

5. Are the discussion and conclusions well balanced and adequately supported by the data? No. The variability of the increase in MMP9 at 3 months in the Smoker group does support the strong conclusions: “MMP-9 release was unaffected by smoking cessation. Its elevation in IS even after smoking cessation may contribute to ongoing lung damage typical of COPD.” The overall balance of proteinase to antiproteinase levels could be improved because the largest amount of proteinase in these samples was MMP8, which decreases more than MMP9 increases. The conclusion that cessation does not stop the “ongoing lung damage typical of COPD” also is faulty because the subjects included COPD (n = 7) and non-COPD subjects (n=10). Lastly, what happen to the decline in lung functions of the individual who stopped smoking, did they continue to progress?

Correct. However, it is known that different inflammatory cells or epithelial cells express different MMPs and the localization of expression varies. The protective role of MMP-9 is based on some earlier studies, but this aspect has been discussed as suggested in more detail. Couple of months is too short to evaluate the overall changes in lung function (this has been added to the revised manuscript).

7. Do the title and abstract accurately convey what has been found? No. The title “Matrix metalloproteinase-9 mediated inflammation…” implies that MMP9 is the cause of inflammation. However, inflammation (i.e. neutrophil levels) in the smoker declined at 6 months whereas the MMP9 levels did not.
Again, the conclusion of the abstract is too strong.

We totally agree, for some reason the “wrong title” was in the final version, it had been corrected once before the final submission. We are sorry about this. The title has been changed to “Matrix Metalloproteinase-9 Elevation in Sputum specimens Persists up to 6 Months after Smoking Cessation” and the conclusion in abstract is also modified.

Minor/Discretionary Concerns
1. It is difficult to see the median line in the solid boxes of the Nonsmoker in Figure 1.

It is true, we made new figures with individual dot plots which are easier to interpret. The absolute levels of the neutrophils, MMPs and TIMP-1 in non-smokers are now included in the text (otherwise there are too many Figures for this short paper).

2. Do the authors clearly acknowledge any work upon which they are building, both published and unpublished? Somewhat

The bibliography is sparse and could be improved by including information on the benefits of smoke cessation. In addition, endogenous activation of MMP9 and MMP14 in non-smoking persons with asthma and COPD due to endogenous acrolein formation has been reported in the literature. This could explain the persist MMP9 levels.

This is an important topic. We added a few sentences about the benefits of smoking cessation in the Discussion section and also that the disease itself can induce MMP expression with appropriate references. However, there are hardly any studies how smoking cessation effects on various MMP levels in sputum of smokers.

3.6. Are limitations of the work clearly stated? Yes.

However it would nice if the paper stated that 6 months may not be long enough of a follow-up. Most benefits for smoke cessation (reduced lung cancer risk or slowing of loss of lung function) only are measurable after 3 years.

Yes this is true and is now added to the Discussion section with appropriate references.
Reviewer: Louise E Donnelly
Reviewer's report:

Major compulsory revisions
1. Although expression of MMP is very important, the activity of these enzymes is critical to understanding their role in disease pathology. Do the ELISA assays used in this study measure both active and latent forms of the enzymes? If so, this should be discussed and where possible activity assays performed. In addition, do these assays measure TIMP associated MMP? These are important points that should be addressed.

ELISA assay is based on immunoreactivity, not the activity. However, ELISA system is also widely used in previous studies. Moreover in many corresponding studies on sputum MMPs (also in our recent study) MMP-ELISA levels have been found to correlate with the activity at least in case of MMP9 (gelatinase). A few sentences about this have been added to the Discussion section.

2. A table of differential and total cell counts for the sputum samples should be included. This is important particularly for the baseline data for the three subgroups.

This is a good point. We added the percentages and total counts of neutrophils in the results section. Also the levels of MMPs and TIMP-1 in non-smokers are now in the text. We did not include any further Tables or Figures (for non-smokers) since the paper is short.

3. Fig 1 might be better presented if data were displayed as dot plots. This would allow the reader to see the ‘n’ in each of the groups.

The Figure has been corrected (and the values of non-smokers removed to the text).

4. The data presented for MMP-9 would indicate that after 3 months smoking cessation, expression of this enzyme increases but then decreases at 6 months. Is this correct? If possible, a separate analysis for each of the nine subjects that quit smoking for 6 months should be performed.

This was unclearly explained but is now corrected. MMP-9 increased after 3 months of smoking cessation and was still significantly elevated at 6 months after smoking cessation when compared to the baseline.

5. The discussion should contain some reference to activity or otherwise of MMP and what is actually being measured in this study.

This is a good point and this topic is now discussed in the Discussion section with appropriate references.

Minor essential revisions
1. MMP-7 (matrilysin) may act to breakdown elastin but is not considered an
elastase as described in the introduction (page 4, line 7) and is not as highly expressed as other MMP in human disease. This sentence should be amended.

We agree. “Elastase” is removed and replaced with “acts to breakdown elastin”.

2. Concentration of DTT used to process sputum and the final concentration of DTT should be included in the methods section (page 6, line 3).

This was missing, but four volumes of dithioerythritol (DTE) were added as the weight of the sample. This is now added to the methods section.

3. Centrifugation should be described in terms of ‘g’ not rpm (page 6, line 6)

Since in previous studies centrifugation is described also in terms of rpm (using the same Cytocentrifuge), the term was not changed.