Author's response to reviews

Title: Differential Expression of C-Reactive Protein and Serum Amyloid A in Different Cell Types in the Lung Tissue of Chronic Obstructive Pulmonary Disease Patients.

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Author's response to reviews: see over
Title: Differential Expression of C-Reactive Protein and Serum Amyloid A in Different Cell Types in the Lung Tissue of Chronic Obstructive Pulmonary Disease Patients.

We are grateful for the Reviewer’s comments and queries, which helped us to improve the manuscript substantially. Below, we itemized our response to the comments.

Reviewer 1 Comments: Stelios Loukides

Major comments

1. The main limitation of the current study is the fact that both surgery and Lung cancer might be biases in the whole study design. The authors must clearly state the above issue in the discussion.

REPLY. We thank Prof. Loukides for the cogent comment. Although in this report we were unable to enroll cancer-free control subjects, we recognize that the addition of such a group would have considerably strengthened our study. All of the study participants underwent surgery because of a suspected malignancy. In general, the collection of large biopsy specimens in subjects who are operated for reasons other than suspected cancer is problematic. In order to reduce the impact of this potential confounder on our findings, potential participants were selected from subjects included in a surgical waiting list because of the presence of a suspected malignancy. Consequently, the study population was homogenous for two factors (i.e., presence of suspected lung cancer and collection of surgical lung samples). Nonetheless, 8.1% of the study patients were found to have lesions that were either histologically benign or non-neoplastic in nature. We did not find significant differences in gene expression values between cases with malignant vs non-malignant disease. Although the sample size was too small to allow firm conclusions on the influence of malignancies on the expression of inflammatory molecules in COPD patients, our pilot data seem to exclude a major role played by the presence of neoplasms. We believe that the results of our study can be applied only to patients with COPD and lung malignancies with a similar location. In order to address the Reviewer’s concerns, we have added a comment on this issue on page 13.

2. Regarding the statistical analysis and particularly the correlation procedure I would like to state the following: I would prefer from the authors to perform a regression analysis in order to adjust their relationships for the underlying co-variates and particularly the disease severity, the Charlson index as well as BMI, gender and many others. The latest issue is somehow crucial since many variables are implicated in the systemic inflammatory syndrome and all these variables must be taken into account by the authors.

ANSWER: Many thanks for raising this interesting point. The relationship of systemic inflammatory load with BMI and comorbidities has been the subject of intense investigation. However, few data are available on the impact of comorbidities on the local expression of inflammatory biomarkers. We believe that the sample size in our study is too small to allow a regression analysis as that suggested by Prof. Loukides. In addition, the inclusion of all cell types and biomarkers as potential predictors/covariates would make the results difficult to interpret. That being said, we have run the requested multivariate analysis using gender, BMI, and the Charlson index as covariates (i.e., the main variables found to be significantly different between COPD and controls). After the inclusion of such potential confounders, the results did not appreciably change. We believe that the additional suggested analysis may contribute to the growing debate on gene expression of lung biomarkers and their systemic consequences. Therefore, we have added the requested analysis and its results in the methodology (page 10), results (page 12), and discussion (page 16) per the suggestion.

3. The authors need to clarify the term impairment. Instead of presenting their results
by characterizing patients as mild or moderate impaired they have to provide values for the FEV1 % pred.

ANSWER: Many thanks for the comment. We have added the requested information per the suggestion.

4. As they stated in the methods section 8% of their COPD population did not have malignant disease. It could be interesting to compare those people with those with malignant disease despite the fact that they are so few for statistical interpretations.

ANSWER: The reviewer is correct in pointing out that 8.1% of the study patients were found to have lesions that were either histologically benign or non-neoplastic in nature. Although we did not find significant differences between cases with malignant vs non-malignant disease, we believe that the sample size was too small to draw firm conclusions. In order to address the Reviewer’s concerns, we have revised the text on page 11 that now reads as follows: “Gene expression was not related to the presence of a neoplasm, the tumor type, TNM staging, inhaled corticosteroid intake, degree of lung function impairment in COPD or presence of a positive microbiological culture”.

5. Another important point is whether the presented results are similarly expressed in the systemic circulation. The authors need to comment on that.

ANSWER: We appreciate the insightful comment. Although in this study we did not specifically measure the systemic inflammatory load, one of the most obvious explanations for the presence of systemic inflammation in COPD is that local inflammatory processes in the lung may cause a “spills over” of proinflammatory molecules into the systemic circulation (Sinden NJ, Stockley RA, Thorax 2010). However, the results of previous studies do not completely support this hypothesis. Although proteins originating from the lung may exert systemic effects, no correlations were found between airway cytokine concentrations and their corresponding systemic levels (Vernooy et al., AJRCCM 2002). Even though a correlation between serum and tissue expression should be obtained to support the “spill over” hypothesis, mRNA quantification cannot provide reliable information as to whether: a) the mRNA will be translated into a protein, b) a functional protein will be translated, and c) if such a protein will be finally released into the circulation (Bustin et al., Clin Chem 2009). It is also noteworthy that mRNA and protein concentrations do not always correlate (Gygi et al., Mol Cell Biol 1999). Being this issue so scientifically interesting, we have added a specific comment on page 16 per the suggestion. That being said, our current findings seem to suggest that epithelial cells can play a major role in the pathogenesis of COPD. This is an interesting finding because one of the potential mechanisms contributing to airway fibrosis is the transition of airway epithelial cells to a mesenchymal phenotype expressing myofibroblast characteristics and capable to migrate into the lamina propria. Such a process has been termed epithelial mesenchymal transition (EMT) (Sohal et al., Respir Res 2011). When this phenomenon is accompanied by angiogenesis, it can explain the increased risk of malignant transformation predominantly observed in the large airways of COPD patients (Wang et al., Respir Res 2013). The potential role played by EMT in the pathogenesis of COPD is currently a very active area of research (Sohal et al., Respirology 2010). Although this study was not specifically focused on this issue, we believe that our manuscript may contribute to the scientific debate in the field. We have commented on these points on page 15 per the suggestion.

6. Patients are statistically significant different in regard to the presence of co-morbidities compared to normal subjects. If we consider that co-morbidities are somehow trigger the systemic inflammation then the authors must explain whether the above difference could influence their results.
REPLY. Thank you for raising this interesting point. The presence of differences in terms of comorbidities is a characteristic feature of COPD, i.e. not all patients share the same number of comorbidities (that may also differ in terms of severity). Moreover, several studies have shown that COPD is a systemic disease with important consequences. As discussed above, we believe that the differences in the local expression of inflammatory markers do not reflect the presence of comorbidities. In support to our hypothesis, we have run a multivariate analysis using gender, BMI, and the Charlson index as covariates (i.e., the main variables found to be significantly different between COPD and controls). After the inclusion of such potential confounders, the results did not appreciably change. Therefore, we have added the requested analysis and its results in the methodology (page 10), results (page 12), and discussion (page 16) per the suggestion.

Minor comments

They have to provide in table 2 the FEV1/FVC ratio.

ANSWER: The requested information has been added per the suggestion.
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We are grateful for the Reviewer’s comments and queries, which helped us to improve the manuscript substantially. Below, we itemized our response to the comments.

Reviewer 2 Comments: Maarten van den Berge

Major comments

1. With respect to the differences between COPD and non-COPD. Do the authors also have data on the total number of cells available in lung tissue? That might be of help in interpreting the data. If not available, it may be a good idea to explain why this could not be a confounding factor.

REPLY. Many thanks for raising this interesting point. In general, the surgeons were instructed to obtain macroscopically similar tissue samples from both COPD and non-COPD subjects. Although we did not measure the exact number of cells, cell purity was determined by flow cytometry in all of the samples. The results indicated cell purity values close to 90%, with no major differences among samples. In order to address the Reviewer’s concerns, we have added a specific comment on this issue (please see page 9).

2. Do they also have data on systemic inflammation available. If so, this should be included in the manuscript.

REPLY. We are grateful to the Reviewer for the cogent comment. The relationship between local and systemic inflammation is an interesting research topic in the field of COPD and this is an active line of investigation for our research group. Unfortunately, we did not specifically measure the systemic inflammatory load for the purpose of this study. In order to address this issue, we have added a comment in the “Discussion” section (please see page 16).

3. How is the inflammation in lung tissue related to severity of COPD?

REPLY. In our study, we did not detect any significant difference between this small group of patients and the rest of the study population. However, such a negative finding may be ascribed to the small sample size that may not allow reaching reliable conclusions. In order to make the point clearer, we have modified the text on page 11, as follows: “Gene expression was not related to the presence of a neoplasm, the tumor type, TNM staging, inhaled corticosteroid intake, degree of lung function impairment in COPD or presence of a positive microbiological culture”.

4. The authors show differences in inflammatory genes within the different inflammatory cell types in lung tissue. Some are increased in COPD vs non-COPD. Others are decreased. Although systemic inflammation is important, the clinical implications (or possible clinical implications) of these particular findings remain unclear. Which cell type is most relevant for systemic inflammation in COPD? Can it be treated or targeted? This should be better explained in the discussion section.

REPLY. Many thanks for the insightful comment. Although in this study we did not specifically measure the systemic inflammatory load, one of the most obvious explanations for the presence of systemic inflammation in COPD is that local inflammatory processes in the lung may cause a “spills over” of proinflammatory molecules into the systemic circulation (Sinden NJ, Stockley RA. Thorax 2010). However, the results of previous studies do not completely support this hypothesis. Although proteins originating from the lung may exert systemic effects, no correlations were found between airway cytokine concentrations and their corresponding systemic levels (Vernooy et al., AJRCCM 2002). Even though a correlation between serum and tissue expression should be
obtained to support the “spill over” hypothesis, mRNA quantification cannot provide reliable information as to whether: a) the mRNA will be translated into a protein, b) a functional protein will be translated, and c) if such a protein will be finally released into the circulation (Bustin et al., Clin Chem 2009). It is also noteworthy that mRNA and protein concentrations do not always correlate (Gygi et al., Mol Cell Biol 1999). Being this issue so scientifically interesting, we have added a specific comment on page 16 per the suggestion. That being said, our current findings seem to suggest that epithelial cells can play a major role in the pathogenesis of COPD. This is an interesting finding because one of the potential mechanisms contributing to airway fibrosis is the transition of airway epithelial cells to a mesenchymal phenotype expressing myofibroblast characteristics and capable to migrate into the lamina propria. Such a process has been termed “epithelial mesenchymal transition” (EMT) (Sohal et al., Respir Res 2011). When this phenomenon is accompanied by angiogenesis, it can explain the increased risk of malignant transformation predominantly observed in the large airways of COPD patients (Wang et al., Respir Res 2013). The potential role played by EMT in the pathogenesis of COPD is currently a very active area of research (Sohal et al., Respiratory 2010). Although this study was not specifically focused on this issue, we believe that the publication of our manuscript may contribute to the scientific debate in the field. We have added this comment on page 15 per the suggestion.

Minor comments.
5. Table 2. Comorbidities. Which co-morbidities? This may be important in the context of systemic inflammation.

REPLY. In our study, comorbidities were assessed using the Charlson index. The Charlson index is commonly used to investigate the impact of several comorbidities on survival (Charlson M, et al. J Chron Dis 1987). Clinical conditions and associated scores are as follows:

- 1 point: Myocardial infarct, congestive heart failure, peripheral vascular disease, dementia, cerebrovascular disease, chronic lung disease, connective tissue disease, ulcer, chronic liver disease, diabetes.
- 2 points: Hemiplegia, moderate or severe kidney disease, diabetes with end organ damage, tumor, leukemia, lymphoma.
- 3 points: Moderate or severe liver disease.
- 6 points: Malignant tumor, metastasis, AIDS.

The most common comorbidities (apart from respiratory diseases and neoplasms) observed in our cohort were the following: diabetes (16 patients), cardiovascular diseases (10 patients), peptic ulcer disease (7 patients), and liver disease (7 patients). This information has been now reported on page 11 per the suggestion.

Statistical approach. Why the cut-off for alpha of 0.1? This is very unusual even when not taking into account the fact that the authors do not correct their findings for multiple testing. Why not use an alpha of 0.05?

REPLY. Thank you for raising this interesting point. We are aware that a cut-off alpha value of 0.05 is the standard threshold for statistical significance, although some controversy exists on the use of this criterion especially in presence of small sample sizes (Alves G, Yu YK. PLoS One 2014). However, we entirely agree that the use of a standard threshold may facilitate comparison with future studies. Consequently, in the revised version of our paper we have now used the standard alpha of 0.05.