Author's response to reviews

Title: Cytokine gene polymorphisms and serum cytokine levels in patients with idiopathic pulmonary fibrosis

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Author's response to reviews: see over
Dear Dr. Sands,

It is my pleasure to submit the revised version of our manuscript. Based on the reviewers’ suggestions, we have substantially revised the manuscript. The changes made in response to the reviewers’ comments are highlighted in yellow. In addition, per the suggestions of Dr. Saeed Daneshmandi and Dr. Grethe Neumann Andersen, we have added additional tables (for a total of seven tables) to provide more details on the investigated cytokine gene polymorphisms and show new significant findings on the physiological parameters and computed tomography scores.

We thank the reviewers for their valuable comments. We believe that we have adequately addressed their concerns in the revised manuscript, and include point-by-point responses to the comments below.

Should you or the reviewers have any questions, we will be happy to answer them.

Yours sincerely,

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Reviewer: Saeed Daneshmandi

Dear Dr. Daneshmandi,

Thank you for reviewing our study. Your valuable comments have led to significant improvements in our manuscript. We are delighted to respond to your comments below.

Comment 1: Sample size of 60 patients is a low number sample size and insignificant data may be due to low number of studied patients. Especially when comparison of several alleles and genotypes on a chromosome is considered low number of samples could not be able to show the exact correlations. So if it is possible authors increase the number of patients.

Response:
We agree with your comment. However, idiopathic pulmonary fibrosis (IPF) is a rare disease, meaning that it would be extremely difficult to increase the sample size, and it would require many years to enroll the desired number of patients.

Comment 2: There are no data for analysis of Allele differences. Please analysis Allele distributions in Patients and Controls.

Response:
Thank you for pointing this out. We now provide the allele distributions for patients and controls in revised Tables 3 and 4.

Comment 3: Authors can evaluate correlation of partial data such as PFT with distribution of polymorphisms in there patient. It may have some valuable data. For a sample authors can see article below: Mol Biol Rep. 2012 Feb;39(2):1845-53.

Response:
Thank you for the suggestion and for providing this valuable reference. Indeed, using the methods suggested in the cited article, we were able to identify significant associations between the studied genotypes and computed tomography scores.

Comment 4: In Results section please mention the P value amount for all significant and insignificant data. There is no P value for insignificant data in Result section.

Response:
As requested, p-values have been added to the Results section of the revised manuscript.

Comment 5: Deviations from expected Hardy–Weinberg were not calculated.

Response:
We now provide the requested information in Table 2 of the revised manuscript.

Comment 6: How the patients were selected for cytokine ELISA would be clearly mentioned in text and also legend of cytokine levels Table.
Response:
Due to resource limitations, we obtained blood samples from the first consecutively enrolled IPF patients (n = 38) and healthy controls (n = 36). We have added this statement to the serum cytokine assay section (pg. 9) of the revised manuscript. We have also included the cytokine levels in the legend to Table 7, as requested.

Comment 7: Statistical method would be mentioned in Table legends.

Response:
As requested, we now mention the utilized statistical methods in the legend of each Table.

Comment 8: Authors mentioned: “Xaubet and colleagues found associations between TGF-#1 polymorphisms and disease progression in IPF patients [9], but their findings have not yet been validated by other studies, and thus TGF-#1 gene polymorphisms cannot yet be advocated as a genetic marker for IPF.” But authors could review article below for correlation of TGF-β polymorphism with IPF: Chin Med J (Engl). 2011 Jul 5;124(13):1923-7.

Response:
Our statement that TGF cannot yet be advocated as a genetic marker for IPF was meant in relation to disease progression. However, we have modified this sentence based on our new findings. In addition, we now mention the work of Li et al. in our Discussion section (pg. 16).

Comment 9: Why authors selected these SNPs for evaluation in their patients and controls? These polymorphisms affect cytokine production or function? If these SNPs affect cytokine production, the amounts of cytokine serum levels in healthy control subjects were different in different cytokine allele and genotypes?

Response:
The investigated cytokine genotypes were selected based on their associations with cytokine production capacity. In the context of TGF-β1, genotype T/T G/G was characterized as a high producer, T/C G/C was considered an intermediate producer, and C/C G/C was taken as a low producer (as described in the protocol provided with the commercial kit used to assess this TGF-β1 polymorphism). The genotype for each serum cytokine was investigated to evaluate the cytokine production potential of each individual. In Table 7 of the revised manuscript, we now show the median serum cytokine levels with respect to each genotype and allele.

Comment 10: Needs some language corrections before being published

Response:
We believe that the revised manuscript addresses the above concern.
Reviewer: Grethe Neumann Andersen

Dear Dr. Andersen,

Thank you for reviewing our study. Your feedback has helped us significantly improve our manuscript. Furthermore, we greatly appreciate the time and effort you put into calculating the alleles and haplotypes, which aided us tremendously. We are delighted to respond to your comments, as follows.

Comment 1: The discussion is too long and should be appreciably condensed. The possibility that IL-10 may be produced by regulatory T cells should be discussed. Moreover, the frequencies of the gene polymorphisms (IL-6 -174 and TNF# -308) have been examined by Pantelidis et al in a British population. The results as to the gene polymorphisms presented herein should be discussed in relation to results from similar studies in other populations.

Response: As requested, we have condensed the Discussion section, and now mention that IL-10 could be produced by regulatory T cells. Regarding comparison with the study of Pantelidis et al., we have included the information pertaining to our study in the paragraphs on IL-6 (pg. 15) and TNF (pg. 18).

Comment 2a: The tables should be improved, In Table 1 the characteristics of the healthy controls should be incorporated, that is their number, sex, age and smoking habits.

Response: As requested, we now include the characteristics of the healthy controls in Table 1.

Comment 2b: Table 2 is difficult to comprehend and needs a major revision, maybe it should be split into 2 or 3 minor tables. In order to make the table more informative and clear, the allele frequencies in the 2 populations (IPF and healthy controls) should be calculated and shown.

Response: Thank you for pointing this out. The information contained in the original Table 2 has been split into Tables 3 and 4 of the revised manuscript, and the allele frequencies of both IPF patients and controls are now included.

Comment 2c: Especially the data on the gene polymorphisms in the promoter region of IL-10 are difficult to understand. For example you have the first genotype (-1082, -819 and -592):GCC GCC and the second: GCC ACC This could also be written as below, where IL-10 1 stands for haplotype 1.

Response: Thank you for the suggestion. We agree that the IL-10 genotypes are somewhat difficult to understand as written. We did not alter this description, however, because this is how it appears in the sheet provided with the utilized kit. Moreover, we have focused on the distribution of genotypes (not haplotypes) for the studied cytokine polymorphisms in IPF patients and controls, as we believe that this will be less likely to cause confusion. Furthermore, we believe that the relationships between serum cytokine levels and the corresponding genotypes (high, low, and intermediate producers), as now shown in revised Table 7, are more relevant than presenting haplotypes.
**Comment 2d:** Moreover, when I calculate the allele frequencies from your table, I find that the frequencies for the C and T alleles at the IL-10 (-819) locus are equal to frequencies for the C and A alleles at the IL-10 (-592) locus. Is this correct or is it an error?

**Response:**
This is correct. The frequencies of the C and T alleles at the IL-10 (-819) locus are equal to the frequencies of the C and A alleles at the IL-10 (-592) locus.

**Comment 3:** It would be nice if you could present the results from your ELISA analysis as a figure, for example as Box plots with 25 and 75 percentiles. However, I understand that many controls had IL-6 levels that were not measurable. However, you may choose to show these as the cut of value or a value just below the cut off.

**Response:**
Given the small number of patients and controls and the marked skewedness of the data, we do not believe that a box plot would be the best choice. However, we have added a figure showing the median values (with ranges) for the studied serum cytokine levels (Figure 1).

**Minor considerations:**

**Comment 4:** In the section on background it is said in line 2 that fibrosing interstitial pneumonia is limited to the lungs. You should omit the phrase “limited to the lungs” as it is obvious that lung diseases are limited to the lungs.

**Response:**
As requested, we have omitted the phrase ‘limited to the lungs.’

**Comment 5:** In the same section, line 11, the serum levels of IL-6 and TGF###(the word serum should be added).

**Response:**
As requested, we have added the word ‘serum’ at the noted position.

**Comment 6:** In the part with the heading: Chest CT: I suppose you mean high resolution (HRCT) scans.

**Response**
We have replaced the abbreviation ‘CT’ with ‘HRCT’ at this point.

**Comment 7:** The number of references should be cut down to 30. The references should be edited according to international standards.

**Response:**
We have reduced the number of references to 45. We believe that these references are adequate and important to the body of the manuscript. The references are formatted according to the guidelines of *BMC Medical Genetics* and not according to international standards.

**Comment 8:** Needs some language corrections before being published

**Response:**
We believe that the revised manuscript addresses this concern.
Reviewer: Mridula Bose

Dear Dr. Bose,

Thank you for reviewing our study. We are delighted to respond to your comments, as follows.

Comment 1: The power of study with such sample size would negate the detection of any effect of the variants, if any on the population.

Response:
We agree with your comment. However when studying a rare disease like idiopathic pulmonary fibrosis (IPF), it is extremely difficult to increase the sample size, and it could take many years to achieve the desired number of patients. Moreover, published studies on IPF patients from other populations have examined similar or smaller patient groups. These include:

Comment 2: Why the given polymorphisms were chosen is not clear. These are some of the SNPs commonly looked for. Except for this reason no other explanation is provided.

Response:
As indicated in the background material, imbalances between pro- and anti-fibrotic/inflammatory cytokines and growth factors, such as tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta1 (TGF-β1) and IL-6, have been implicated in the pathogenesis of pulmonary fibrosis in other populations. This is why we examined the chosen cytokine polymorphisms for associations with IPF in our Saudi population.

Comment 3: The manuscript doesn’t describe in methods whether proper genetic analysis was done such as checking for HWE, Minor allele frequency. Also, no check for population stratification was included.

Response:
Thank you for pointing this out. We have added a new Table 2 showing our assessment of HWE. In addition, we now provide allele frequencies for patients and controls in Tables 3 and 4. Population stratification is usually not required for this type of study, provided that the controls were randomly selected and the distributions of the observed genotypes do not differ significantly from the expected distribution according to HWE.

Comment 4: The research question and the results are not presented in a lucid manner and the conclusion is also not very clear.

Response:
The manuscript has been revised to clearly state the research question and our conclusions.
Comment 5: There have been studies on serum IL-6 levels and IPF. It is not clear what additional information the presented study adds to the field.

Response:
This is the first study assessing serum IL-6 and IPF in Saudi patients. Furthermore, no previous study has described serum IL-6 in IPF patients with respect to high-, intermediate- and low-producer genotypes. This information may be useful for future studies exploring serum cytokines in terms of genotype-determined production, disease progression and outcome among IPF patients.

Comment 6: Authors could have checked for correlation between the different genotypes and serum cytokine levels between groups. That would have added to some value to the manuscript.

Response:
We did perform correlation analyses, and found no significant association of serum cytokine levels and genotypes with the examined physiological parameters and HRCT scores. However, we have since identified a significant association between the studied polymorphisms and these physiological parameters and HRCT scores. This information is included in the revised manuscript.

Comment 7: The number of samples is too small for any statistical validation. The conclusions drawn are not tenable. The manuscript should be rewritten focusing on the cytokine levels of the patients from the specific region and its possible impact on the management of such patients. The polymorphism of the selected sequences are too general and nonspecific to add any value to either the manuscript or for the local population under study.

Response:
We believe that the revised manuscript addresses all of these points. We believe the findings of the present study offer important insights regarding the relationships between IPF disease severity and the studied genotypes.