Author's response to reviews

Title: Determination of inflammatory biomarkers in patients with COPD: comparison of different assays

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Author’s response to reviews: see over
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Dear Editor of BMC Medical Research Methodology,

Thank you for relaying the comments on our manuscript entitled "Determination of inflammatory biomarkers in patients with chronic obstructive pulmonary disease: Comparison of different assays", by Lopez-Campos et al. We are grateful for the reviewers’ constructive comments and insightful critiques that helped us to improve the manuscript.

We have revised the manuscript according to the reviewers’ comments and suggestions. Please find enclosed the new version of the manuscript, in which all of the suggestions have been incorporated. In particular, we have provided methodological clarifications and updated the discussion with some new references in accordance with the reviewers’ comments. The responses to the reviewers’ comments are also included below.

Thank you very much for your consideration of our paper.

The manuscript has been reviewed by a professional native English speaking editor to ensure that the English language is correct.

Yours sincerely,

Jose Luis Lopez-Campos
Editorial Requests

1. Please include the name of the Institutional Review Board that approved your study.

RESPONSE: The name of my Institutional Review Board is the Comité de Ética e Investigación Clínica del Hospital Universitario Virgen del Rocío, Seville, Spain. I have included this information in the methods sections per your suggestion.

Please also ensure that your revised manuscript conforms to the journal style (http://www.biomedcentral.com/info/ifora/medicine_journals). It is important that your files are correctly formatted.

RESPONSE: We have reviewed the instructions to authors per the suggestion of the Editor.

Reviewer 1.

We are grateful to Dr. Alicia Lacoma for carefully reviewing the manuscript and providing her comments and queries, to which our responses are as follows.

The manuscript is rather a methodological/technical note rather than an original. In fact, the objective of the study is to compare two different assays for the measurement of CRP and SAA. So, no discussion is possible regarding cut off accuracy as no outcome is measured in these COPD patients.

RESPONSE: This is correct. This study analyzed a technique-related question, which influenced our selection of the targeted journal. We aimed to compare both assays and their ability to measure both acute phase reactants in COPD. Biomarkers of COPD, especially CRP, are studied using different techniques to provide prognostic benefit. In this scenario, it is important to clarify the effect of the laboratory technique on the results presented. We believe that our study will provide new information for investigators interested in systemic biomarkers of COPD. We did not aim to study cut-off values. However, because of the discrepancy that we
observed, we considered that we should give the reference value of our cohort, following our own recommendation that we discuss in the present paper.

I agree with the authors that it is important to standardize biomarkers commercial kits. Authors should mention that the standardization of technical assays is necessary for all biomarkers studies in general, not only for COPD so cut offs values are comparable between studies. In fact, the usefulness of systemic biomarkers has been evaluated in several clinical settings (emergency room, hospitalized, intensive care unit) and different diseases, so authors should emphasize that this standardization is not only for stable COPD patients.

RESPONSE: We agree with the reviewer and have added this text in the discussion per the suggestion.

Introduction

Page 3. Please check reference 14, as it is not related to COPD.

RESPONSE: The reviewer is correct. We have deleted this reference per the suggestion.

Material and Methods.

Page 6. Were biomarkers measured at the time of blood collection or were samples stored until measurement? If yes, how samples were stored?

RESPONSE: Samples were stored until measurement and were kept frozen in a -70ºC freezer. We have added this information per the suggestion.

Page 6. Authors refer to sensitivity and limit of detection values for the 4 assays, but which are the values of specificity?
RESPONSE: We apologize for this misunderstanding. We are not referring to the sensitivity in
terms of diagnostic profile, but rather in terms of the limits of detection. We have changed this
aspect of the manuscript to clarify the meaning.

In addition, considering for example CRP, do the authors know if both assays detect the
same fragment of the protein? Are the antibodies the same? Same questions apply for SAA.

RESPONSE: This is a very pertinent comment. We have reviewed the manufacturer’s
instructions have not been able to determine the fragment of the protein that is detected.
However, the findings are based on immunodetection, and they are all obtained from validated
assays that have been used in multiple previous studies. In this regard, it has been
acknowledged that the actual plasma protein that is being measured is the same regardless of

Discussion.

Page 12. Line 2. Please include “…both CRP and SAA levels in comparison to healthy
controls, suggesting…”

RESPONSE: We have changed this sentence per the suggestion.

Page 12. Please remove “recent work” when referring to a study published in 1983
(reference 26).

RESPONSE: The reviewer is correct. We have changed this sentence per the suggestion.

Page 13. Please, could authors update bibliography referring to nephelometry and its
correlation with other techniques? The only reference is from 1981.
RESPONSE: Nephelometry is a laboratory technique that has been discussed in publications since 1948. We have added two new references from previous comparative studies. Interestingly, another recent study on cerebrospinal fluid IgG determination found discrepancies between nephelometry and immuno-enzyme techniques (Keir G, et al. Ann Clin Biochem 2008). We have added these references per the suggestion.

Has the agreement of nephelometry with ELISA been investigated in diseases different than COPD? Which were the results obtained?

RESPONSE: To our knowledge, no studies have assessed the degree of agreement between both techniques in the evaluation of acute phase reactants. In addition to the study by Ker et al., which was already mentioned in the previous response, two studies have evaluated immunoglobulins. The first compared both techniques in the determination of rheumatoid factors (Adebajo AO, et al. Scand J Rheumatol 1992) and showed a better prognostic profile for ELISA. However, the authors compared both techniques by simple linear correlation; interpretations based on this method are largely limited, as explained in the next comment.

Another study performed in Spain (Ginel PJ, et al. Eur J Clin Chem Clin Biochem 1997) compared both methods for detecting the concentrations of immunoglobulins in dogs and showed some differences that favored different techniques depending on the immunoglobulin that was being measured. However, the authors again used a simple linear correlation to study both techniques (please see next comment).

Page 13. If authors state that “...the agreement between the two assays is remarkably low”, it is not possible that later in the discussion they state “Although the two assays correlate well, they may provide different information in COPD...”. I do not understand how both assays might provide different information, if they measure the same biomarker. Please explain and discuss.
RESPONSE: We thank the reviewer for this very pertinent and interesting question. When the agreement between the two methods of measurement is studied, the first step for the researchers is to generate a graph of points of the dispersion of the present data with both techniques. The second step is usually to calculate the correlation coefficient \( r \) between the two methods. The null hypothesis here is that the measurements of the two methods are not linearly related. If the probability is very low, we can safely conclude that both measurements are related, as was demonstrated years ago for CRP and SAA, which are both major acute-phase reactants (Raynes JG, Cooper EH. J Clin Pathol 1983).

However, this correlation does not mean that the two methods agree in their measurement (Bland JM, Altman DG. Lancet 1986). In this sense, an understanding of the difference between correlation and agreement is important for the consideration of the following arguments.

First, the simple linear correlation coefficient \( r \) measures the strength of a relationship between two variables and not the agreement between them. In this regard, a change in measurement scale does not affect the correlation, but it certainly does affect the agreement. It is then possible that two measurements are correlated and that one increases as the other increases. However, if, for instance, different units of measure are used, then the techniques will not be measuring the same outcome, although they may correlate.

Second, the correlation depends on the size of the sample, whereas the agreement does not. If the sample size is large, the correlation will be greater than if the sample size is small. Because researchers are trying to compare the two methods across the range of values that are normally found, a high correlation is almost guaranteed. Finally, statistical significance tests that study two methods are usually related because it would be surprising if two methods designed to measure the same quantity were not correlated. Therefore, the significance of a correlation test is irrelevant to study the agreement between two measures. In our manuscript, we studied the degree of agreement using a proper statistical approach with the Bland-Altman graphs. Thus, we affirm that the two assays correlate well because they have a high correlation coefficient. However, they do not agree with regard to their measure, i.e., providing different information as briefly explained in the discussion (page 13). We do not provide more of this statistical
explanation so that we may focus more on the technical aspects of the study, but we can expand the explanation if needed.

Page 14. Line 5. In relation with the previous comment, how this difference is likely to be clinical significant?

RESPONSE: We thank the reviewer again for this very appropriate question. We have tried to respond to this question in the discussion on page 14. The response is in relation to threshold values. However, the threshold values for these biomarkers have been scarcely studied. The only study that addressed this question with a proper statistical approach was that of Broekhuizen et al. (Thorax 2006), who identified a reliable cut-off for increased CRP concentrations of 4.21 mg/L in patients with chronic respiratory disorders. However, the value of 3.0 mg/L has been extensively used.

Regardless of which cut-off value we consider, a change in 3.9 units for CRP in COPD would probably exceed the threshold value. Thus, a patient could be above or below this clinically prognostic cut-off value, which may or may not suggest a clinical prognostic effect depending on the technique used for the determination. The same argument can be applied to SAA.


RESPONSE: “Heretofore” is an English expression that means “until now”.

A “Limitations” section is missing.

RESPONSE: The main limitation of the study is the lack of a prospective follow-up to determine whether these differences are relevant in the long-term. A long-term follow-up of the patients would have been useful if the differences in the measurements are relevant from a clinical point of view in the long term. However, the methodology used did not include this follow-up. We have included this information in the discussion per the suggestion of the reviewer.
Considering both biomarkers and referring only to the technical information, ELISA tests are more sensitive than nephelometric assays. So, it is reasonable to expect that levels measured with ELISA will be lower, regardless of patient group. In addition, it has already been demonstrated that COPD is characterized by an increase of systemic inflammation, in comparison to healthy controls. So, I cannot really see which were the results authors expected.

RESPONSE: We are not sure of what the reviewer is asking. With this study, we expected that both laboratory techniques were similar, such that either could be used for the determination of systemic inflammatory biomarkers in COPD. Our findings were contrary to our expectations, and we believe that it is important that the members of the scientific community who are involved in the evaluation of COPD biomarkers have this information for future studies.

Although no statistical differences were found, authors should discuss the possible influence of corticosteroids on CRP levels, as different published studies have found controversial results on this issue.

RESPONSE: The reviewer is correct; this is a controversial issue. The relationship between inhaled corticosteroid use and the systemic inflammatory load in COPD is a subject of debate. In an initial small study, the use of inhaled corticosteroids with a dose of 1,000 µg of fluticasone per day was effective in reducing serum CRP levels in patients with COPD. However, several years in a clinical trial multicenter study with a larger sample size, the same group found that this association was not present for CRP. The present study failed to show this association, and our results provide further evidence for this lack of association. We have added this information to the discussion per the suggestion.

Authors should discuss the relationship between CRP and SAA synthesis, as both of them are acute phase reactants.
RESPONSE: CRP and SAA are both major acute-phase reactants in humans. Both are synthesized in the liver by factors that initiate and maintain the inflammatory response, such as interleukin (IL)-1, IL-6, and tumor necrosis factor α. Although several inflammatory mediators induce and control the secretion of these biomarkers, CRP is predominantly induced by IL-6, whereas SAA is stimulated mainly by IL-1. We have included this information in the discussion per the suggestion.

Tables/ Figures

I recommend keeping only Figure1, Table 1, Table 2 and Table 3. Authors should remove Figure 1 to Figure 7 (foot figures) and instead include Panel a, b, c and d respectively, as necessary. Please correct also in the text.

RESPONSE: We are confused about this comment. The figures are already organized as 1 to 3 with panels labeled from a to d. We have eliminated Figure 2 per the suggestion. However, figure 3 is related to the Bland-Alman analysis, and we strongly suggest that it should be kept. If after reading our responses, the reviewer still recommends the elimination of figure 3, we shall proceed accordingly.

Table 3. Check value for SAA in controls.

RESPONSE: We have reviewed table 3, and the values are correct. Please note that these values represent differences between techniques and not the actual concentration values of the molecules that were studied. Because we subtracted the nephelometry values from the ELISA values, the data indicate that the values obtained using nephelometry were significantly higher than those obtained using ELISA for both CRP and SAA. The mean for SAA was negative for the controls, which indicates that this relationship was generally inverted. However, the standard deviation should also be considered here.
Reviewer 2.

We are grateful to Prof. Emiel Wouters for carefully reviewing the manuscript and sending his comments and queries, to which our responses are as follows.

It remains unclear why the authors have not chosen a disease independent approach to answer their methodological hypothesis;

RESPONSE: This is an interesting comment. Prof. Wouters is right in suggesting that a disease-independent approach would have given us a broader picture of the behavior of both techniques. However, CRP has been a source of investigation for COPD in recent decades, and the difference encountered is especially relevant in this disease. The results of our study showing increased differences in COPD compared with controls confirm our approach. Therefore, investigators evaluating these biomarkers in the future should be aware of this difference.

Furthermore, it would be very interesting to become informed about the variability and reliability of both markers assessed by both methodologies.

RESPONSE: We thank Prof. Wouters for this interesting and appropriate comment. Variability and reliability include studying the variation of both biomarkers over time to assess whether the concentration of the proteins and the ability of both techniques to detect the variations change over time. Interestingly, the repeatability of blood biomarkers over time has been recently reported for the ECLIPSE cohort (Dickens JA, et al. Respir Res 2011). However, the present study employed a cross-sectional design, and we did not perform a follow-up of the patients. This is a limitation of the study, and we have included this limitation in the discussion section.

This methodological study is integrated into a second study evaluating systemic inflammation assessed by CRP and SAA in COPD patients. The finding of increased CRP and SAA levels in COPD versus smoking controls offers limited new information. In order
to evaluate this hypothesis, the study has limitations in terms of lack of age matched non-smoking controls, matching of smoking controls with the COPD population.

RESPONSE: This is an interesting point. The reviewer is right in pointing out that the elevation of these biomarkers has already been reported, with a considerable number of publications for CRP but much fewer publications for SAA. However, we would like to emphasize that the aim of our study was to compare both laboratory techniques. In our case, mean CRP and SAA levels did not differ significantly between smokers and previous smokers, and so we did not further explore this relationship in our cohort. Therefore, we concentrated on the differences between the techniques. Nonetheless, we agree that a larger study with a larger sample size and a long-term follow-up would shed some light on the behavior of these biomarkers, especially that of SAA, which has been scarcely explored.