High levels of circulating osteopontin in inflammatory lung disease regardless of Sars-Cov-2 infection.

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)
Dear Dr. Cappellano,

Thank you for the submission of your manuscript to EMBO Molecular Medicine. I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

1) Please address all referee's comments.
2) Figures: Please upload individual, high-resolution figure file and name it Figure 1 also in the main text. Figure legend should remain at the end of the main manuscript text. For more information on figure presentation please check "Author Guidelines".
https://www.embopress.org/page/journal/17574684/authorguide#datapresentationformat
3) In the main manuscript file, please do the following:
   - Add up to 5 keywords.
   - Add contributions for all authors.
   - Conflict of interest statement should be named "Conflict of interest".
   - Include a statement that informed consent was obtained from all human subjects and that, in addition to the WMA Declaration of Helsinki, the experiments conformed to the principles set out in the Department of Health and Human Services Belmont Report.
4) Funding: Please make sure that information about all sources of funding are complete in both our submission system and in the manuscript.
5) As part of the EMBO Publications transparent editorial process initiative (see our Editorial at http://embomolmed.embopress.org/content/2/9/329), EMBO Molecular Medicine will publish online a Review Process File (RPF) to accompany accepted manuscripts. This file will be published in conjunction with your paper and will include the anonymous referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript. Let us know whether you agree with the publication of the RPF and as here, if you want to remove or not any figures from it prior to publication. Please note that the Authors checklist will be published at the end of the RPF.
6) Please provide a point-by-point letter INCLUDING my comments as well as the reviewer's reports and your detailed responses (as Word file).

I look forward to reading a new revised version of your manuscript as soon as possible.

Yours sincerely,

Zeljko Durdevic
Referee #1 (Remarks for Author):

In their correspondence "High levels of circulating osteopontin in inflammatory lung disease regardless of Sars-CoV-2 infection." the authors test osteopontin as a biomarker for SARS-CoV-2 infection. The rationale is based on a report by Gibellini et al. published in 2020 addressing monocyte alterations in SARS-CoV-2 and showing increased osteopontin levels in COVID-19 patients.

While Gibellini et al. had compared COVID-19 patients to healthy controls, the authors of this manuscript test whether increased osteopontin is specific for SARS-CoV-2 infection as opposed to other causes of pneumonia, which indeed turned out not to be the case. Even though this might not necessarily be surprising, it is an interesting finding worth reporting. Not least does it demonstrate that findings made with respect to COVID-19 need to be put into context and it needs to be discriminated between what is more general infection-associated inflammation and what is SARS-CoV-2-specific. There are some points to be addressed:

1) As an introduction it is stated that "Gibellini et al. (2020) showed that the bioenergetic alteration of the monocyte compartment affects the pathogenic response to Sars-Cov-2 infection". As such effects on the pathogenic response were not explicitly shown by Gibellini et al. and they rather demonstrated alterations to the monocytic compartment in COVID-19, please revise this introductory statement to more precisely reflect the findings of Gibellini et al.

2) "OPN is a multifaceted molecule involved in the inflammatory response: it modulates leukocyte activation, migration and differentiation and induces cytokine secretion both in acute and chronic inflammation.". Please provide a reference for the effects of OPN.

3) As OPN is affected in a broad array of conditions, the authors might want to indicate their criteria defining healthy controls.

4) Regarding the cohort of Sars-Cov-2 negative patients if possible please indicate whether these were viral, bacterial, any other pneumonia patients or how these characteristics were distributed within the cohort.

5) Even though OPN is not a marker for SARS-CoV-2 infection, could the authors speculate on whether increased OPN might be associated with a certain form or severity of COVID-19. There is quite some heterogeneity in the OPN levels reported in SARS-CoV-2 positive patients. It would be interesting to learn whether e.g. explicitly high levels are associated with severe disease. This might make OPN suitable not as a biomarker for SARS-CoV-2 per se but e.g. for the course of the acute disease or the intensity of inflammation.
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R1. We thank the reviewer for his/her comment, we revised the sentence, as following "Gibellini et al. (2020) showed that the bioenergetic alteration of the monocyte compartment affects the inflammatory response to Sars-Cov-2 infection”.

Q2) "OPN is a multifaceted molecule involved in the inflammatory response: it modulates leukocyte activation, migration and differentiation and induces cytokine secretion both in acute and chronic inflammation.". Please provide a reference for the effects of OPN.

R2. As suggested, by the reviewer, we added the missing reference (Clemente et al., 2016).

Q3) As OPN is affected in a broad array of conditions, the authors might want to indicate their criteria defining healthy controls.

R3. We thank the reviewer for his/her valuable suggestion. We edited the text to clarify it.

Q4) Regarding the cohort of Sars-Cov-2 negative patients if possible please indicate whether these were viral, bacterial, any other pneumonia patients or how these characteristics were distributed within the cohort.

R4. We thank the reviewer for his/her comment. We added the following sentences: ‘Diagnosis at hospital admission were respiratory failure related to acute or acute-on-chronic heart failure (n=17), sepsis (n=16 of which 25% due to community acquired pneumonia), intracranial bleeding (n=8), trauma (n=5) and others (n=10)”.

Q5) Even though OPN is not a marker for SARS-CoV-2 infection, could the authors speculate on whether increased OPN might be associated with a certain form or severity of COVID-19. There is quite some heterogeneity in the OPN levels reported in SARS-CoV-2 positive patients. It would be interesting to learn whether e.g. explicitly high levels are associated with severe disease. This might make OPN suitable not as a biomarker for SARS-CoV-2 per se but e.g. for the course of the acute disease or the intensity of inflammation.

R5. The reviewer is right; we added in the text the following sentences: “OPN deregulation may impact on COVID-19 in different ways: it might either be associated with disease severity or with
the development of a fibrotic phenotype. Several factors in Sars-Cov-2 infection may influence OPN secretion i.e., amount of inflammatory host response, disease progression and therapy prescribed to the patients i.e., anti-inflammatory drugs, corticosteroids and heparin. Noteworthy, in the past we showed that anti-OPN autoantibodies, with neutralizing properties, are spontaneously produced during inflammatory conditions boosting OPN production (Clemente et al., 2017). Differential production of such response, may impact on the level of resolution of the inflammatory burst. Furthermore, due to the multifaceted nature, OPN may also act as a pro-fibrotic protein. Currently, no fully proven options are available for the treatment of post-inflammatory COVID-19 pulmonary fibrosis, since the pathogenetic mechanism is not fully understood. Risk factors include age, history of smoking, severe illness, and prolonged mechanical ventilation, but monitoring OPN (and its autoantibodies) levels at discharge and during COVID-19 follow-up, may be relevant, in view of considering OPN as a potential predictor factor of pulmonary fibrosis. A well-designed study, considering all these variables, is needed to elucidate the association of OPN with disease outcome.”
We are pleased to inform you that your manuscript is accepted for publication and is now being sent to our publisher to be included in the next available issue of EMBO Molecular Medicine.
Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- The data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- Figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- Graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- If n≥5, the individual data points from each experiment should be plotted and any statistical test employed should be justified.
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- A specification of the experimental system investigated (e.g. cell line, species name).
- The assay(s) and method(s) used to carry out the reported observations and measurements.
- An explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- The exact sample size (n) for each experimental group/condition, given as a number, not a range.
- An explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- Definitions of statistical methods and measures:
  - Common terms, such as p-value (please specify whether pairwise or unpaired), simple t-tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section.
  - Are tests one-sided or two-sided?
  - Are there adjustments for multiple comparisons?
  - Exact statistical test results, e.g., P values < x but not P values < x; definition of "center values" as median or average; definition of error bars as s.d. or s.e.m.

Any description too long for the figure legend should be included in the methods section and/or with the source data.

If the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non-applicable).

We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?

The sample size was chosen according to previous studies performed in our lab (Cassetti et al., 2019, Chiocchetti et al., 2004).

1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.

None.

2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?

Inclusion criteria were patients hospitalized with proven Sars-Cov-2 infection (Sars-Cov-2 positive group) and with Sars-Cov-2 negative patients hospitalized with pneumonia-associated symptoms (Sars-Cov-2 negative group). Healthy control were non-coronavirus and non-hospitalized subjects.

3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.

None.

4. a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.

None.

4.b. For animal studies, include a statement about blinding even if no blinding was done.

None.

5. For every figure, are statistical tests justified as appropriate?

Yes, data normality was assessed using D’Agostino-Pearson test, the non-parametric Kruskal-Wallis test with Dunn’s correction was then used.

6. Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.

Yes, data normality was assessed using D’Agostino-Pearson test.

7. In there an estimate of variation within each group of data?

Standard deviation in HC was 14.90, in Sars-Cov-2+ 37.09 and in Sars-Cov-2-: 53.33, Bartlett test p<0.001.

8. What data presentation style was used (e.g. box plots, violin plots, etc.)? Explain any differences in data presentation between groups.

Please fill out these boxes. (Do not worry if you cannot see all your text once you press return.)
**C- Reagents**

8. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodies (see link list at top right), 1Degreeweb (see link list at top right).

9. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.

10. Provide a statement only if it could.

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**D- Animal Models**

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.

9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.

10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'.

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**E- Human Subjects**

11. Identify the committee(s) approving the study protocol.

12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

13. For publication of patient photos, include a statement confirming that consent to publish was obtained.

14. Report any restrictions on the availability (and/or on the use) of human data or samples.

15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.

16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.

17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.

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**F- Data Accessibility**

18. Provide a “Data Availability” section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Expression Omnibus GSE19462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for ‘Data Deposition’.

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**G- Dual use research of concern**

21. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.