EMBO Molecular Medicine

DNA Methylation Changes in COVID-19 Link to Long-Term Transcriptional Dysregulation in Airway Cells

Marey Messingschlager, Sebastian Mackowiak, Maria Völker, Matthias Bieg, Jennifer Loske, Robert Chua, Johannes Liebig, Sören Lukassen, Loreen Thürmann, Anke Seegebarth, Sven Twardziok, Daria Doncevic, Carl Herrmann, Stephan Lorenz, Sven Klages, Fridolin Steinbeis, Martin Witzenrath, Florian Kurth, Christian Conrad, Leif Sander, Naveed Ishaque, Roland Eils, Irina Lehmann, Sven Laudi, and Saskia Trump

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(Note: Please note that the manuscript was previously reviewed at another journal and the reports were taken into account in the decision making process at EMBO Molecular Medicine. Since the original reviews are not subject to EMBO Press' transparent review process policy, the reports and author response cannot be published. 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.)

19th Feb 2025

Dear Dr. Trump,

Thank you for submitting your manuscript to EMBO Molecular Medicine. We have now received the enclosed report from the referee who was asked to assess your work and your response to the reviewers comments from the previous journal. As you will see, the referee thinks the raised concerns have been addressed, and I am pleased to inform you that we will be able to accept your manuscript pending the following amendments:

1. We suggest changing "Dysregulation of Transcriptional Programs" to "Transcriptional Dysregulation" in the title.

2. Please provide a written response to the reviewer's comments regarding the lack of definitive proof of long-term epigenetic modifications in a 'point-by-point response' and discuss this as a limitation.

On a more editorial level, please address the following issues:

1. Main figures should be removed from the manuscript and uploaded as individual, high resolution figure files. The legends should be compiled at the end of the manuscript text.

2. Please provide a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/17574684/authorguide#submissionofrevisions). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the Review Process File (RPF).

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4. Funding information should be merged with Acknowledgements. Complete funding information should be entered into our submission system as well.

5. Appendix: the "Online Methods" (and References) should be removed from the file and added to the "Methods" in the main manuscript text. The supplementary figures should be renamed to "Appendix Figure S1 - S10" and their callouts should also be corrected in the main manuscript text. The files should be uploaded as a PDF, labelled "Appendix", and a table of contents with page numbers should be added to the first page. Please remove the track changes in the Appendix file.

6. Supplementary tables: Table S3, S4, S6, S7, S9, S13, S18 should be renamed to "Dataset EV1" - 7 and uploaded as separate files. Tables S1, S2, S5, S8, S10, S11, S12, S14, S15, S16, S17 should be renamed to "Table EV1" - 11 and uploaded as separate files. The callouts should be updated accordingly.

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- the medical issue you are addressing,
- the results obtained and
- their clinical impact.

This may be edited to ensure that readers understand the significance and context of the research. Please refer to any of our published articles for an example.

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these in a separate file or send them by email, we will incorporate them accordingly.

Please also suggest a visual abstract to illustrate your article as a PNG file 550 px wide x 300-600 px high.

12. All Materials and Methods need to be described in the main text using our 'Structured Methods' format. According to this format, the Methods section includes a Reagents and Tools Table (listing key reagents, experimental models, software and relevant equipment and including their sources and relevant identifiers) followed by a Methods and Protocols section describing the methods, ideally using a step-by-step protocol format. The aim is to facilitate adoption of the methodologies across labs.

Please download and fill our Reagents and Tools Table template (.docx), which you can find in our author guidelines:
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14. Data availability: please add the EGA ID.

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

Kind regards,
Jingyi

Jingyi Hou
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- the results obtained and
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This may be edited to ensure that readers understand the significance and context of the research.
Please refer to any of our published articles for an example.

6) Author contributions: the contribution of every author must be detailed in a separate section.

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Each figure should be given in a separate file and should have the following resolution:
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***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

The manuscript of Messingschlager et al. investigates transcriptomic and epigenetic (DNA Methylation) in samples of the upper respiratory tract after SARS-CoV-2 infection.

The manuscript was submitted to another journal and evaluated by two referees which provided suggestions for improvement.

The authors revised there manuscript and wrote a rebuttal letter both of which were now submitted to EMBO molecular medicine.

The methods and statistical analyses of the data are sound.

A similar longitudinal study with samples of the upper respiratory tract has not been performed (to my knowledge).

There is no mechanistic data, thus the medical impact is difficult to assess.

Model system is human.

Referee #1 (Comments for Author):

I understand that the editor is asking for my opinion on the manuscript in general and the extent to which the authors have addressed the critiques raised by the two referees.

My general comments are listed above. The study is interesting and provides a starting point for a better understanding of post-COVID conditions. It is based exclusively on genomic sequencing data, with no accompanying phenotypic or mechanistic data. However, a novel follow-up cohort was introduced, in which transcriptomic data were correlated with clinical findings.

Overall, the quantity of data added in response to reviewer comments is extensive, and the quality is good. The authors were asked to tone down their interpretations, as there were many overstatements regarding mechanistic findings. This issue has been addressed.

The authors also claim that the DNA methylation observed in this study affects immune regulatory genes and genes associated with ciliary function, with these changes persisting 12 months post-infection. However, the study fails to link these transcriptomic changes with long-term epigenetic modifications, as suggested by the referees. This is surprising, as the quantification of DNA methylation is technically straightforward.

In conclusion, the majority of the critiques raised by the referees have been addressed. However, one key point-providing definitive proof of long-term epigenetic modifications is still missing.

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In conclusion, the majority of the critiques raised by the referees have been addressed. However, one key point-providing definitive proof of long-term epigenetic modifications is still missing.

Thank you for your comment. As described in the manuscript, we were unable to assess DNA methylation levels in the follow-up samples due to the unavailability of remaining cells or DNA from these samples. We acknowledge that linking long-term transcriptomic changes to persistent epigenetic modifications would have been valuable. However, given this technical constraint, such an analysis was not feasible in our study.

To ensure that this limitation is more clearly stated, we have now emphasized it in the discussion (lines 377–379, new text in bold):

"*Another limitation is the absence of mDNA-seq data of the follow-up samples due to a lack of available DNA.* ***Thus, we were not able to analyse DNA methylation and gene expression levels concurrently in the follow-up samples, which limits the direct association to epigenetic changes.***"

Furthermore, in the *Paper explained* section we slightly shifted the focus to the persistent transcriptional dysregulation while acknowledging that these changes are most likely associated with epigenetic perturbations initiated during the acute phase of the disease:

"The data suggest that respiratory post-acute sequelae of COVID-19 may be related to long-term transcriptional dysregulation in airway epithelial cells, likely associated to epigenetic perturbations initiated in the acute phase of disease."

3rd Mar 2025

Dear Saskia,

Thank you for sending us your revised manuscript. I am 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.

Your manuscript will be processed for publication by EMBO Press. It will be copy edited and you will receive page proofs prior to publication. Please note that you will be contacted by Springer Nature Author Services to complete licensing and payment information.

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Should you be planning a Press Release on your article, please get in contact with embo_production@springernature.com as early as possible in order to coordinate publication and release dates.

If you have any questions, please do not hesitate to contact the Editorial Office. Thank you for your contribution to EMBO Molecular Medicine.

Yours sincerely,
Jingyi

Jingyi Hou
Senior Editor
EMBO Molecular Medicine

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Journal Submitted to: EMBO Molecular Medicine	The EMBO Journal - Author Guidelines
Manuscript Number: EMM-2025-21342-V2	EMBO Reports - Author Guidelines
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This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your

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Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

2. Captions

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

- a specification of the experimental system investigated (eg 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 being measured.
- 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;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 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;
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Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

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Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

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Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

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If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Yes	Table 1, Table EV1, Table EV7 and Table EV11

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If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgements

Design

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If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

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Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Methods in paragraph "Study design and participants"
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Yes	Methods in paragraph "Study design and participants"
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Yes	Methods in paragraph "Study design and participants"
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Methods in paragraphs "Processing and analysis of whole genome methylation sequencing data" and "Processing and analysis of single cell 3' RNA sequencing data" (Apart from DSS, which was used for the determination of differentially methylated regions and is adjusted to the bimodal distribution of DNA methylation data, as well as the the MAST test, which is tailored to zero-inflated single cell data, we only used nonparametric tests, such as Wilcoxon rank sum test and Spearman correlation, as the data were not normally distributed.)

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In the figure legends: state number of times the experiment was **replicated** in laboratory.	Not Applicable	
In the figure legends: define whether data describe **technical or biological replicates**.	Not Applicable	

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Ethics	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Yes	Methods in paragraph "Study design and participants"
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Yes	Methods in paragraph "Study design and participants"
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
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Reporting

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Adherence to community standards	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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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.	Not Applicable	
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.	Not Applicable	

Data Availability

Data availability	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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If publicly available data were reused, provide the respective **data citations in the reference list**.	Yes	Data Availability