Author's response to reviews

Title: Influence of condensation temperature on selected exhaled breath parameters

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Author's response to reviews: see over
This manuscript assesses the effect of cooling temperature (four different temperatures: -10, -5, 0, and +5°C) on parameters of exhaled breath condensate such as condensed volume, conductivity, concentration and total amount of hydrogen peroxide (H2O2) and malondialdehyde (MDA). Exhaled breath condensate (EBC) was collected from 24 healthy non-smoking subjects during 10 min. sessions with commercially available device TURBO-DÉCCS (equipped with electronically controlled refrigerating system based on Peltier effect, single use polyethylene device for direct EBC collection, mouthpiece with one-way valve). Each EBC specimen was screened for alpha-amylase activity to exclude the saliva contamination. The methods used for determination of these EBC parameters are adequate and have sufficient sensitivity and preciseness. The authors found that increase in cooling temperature causes the rise of EBC conductivity and H2O2 and MDA concentration. In contrast, this caused the decrease in total condensate volume. By appropriate statistical analysis and calculations authors estimated usefulness of conductivity as a possible normalization factor and assessed ex vivo volatility of H2O2 and MDA. Finally, authors propose recommendations for EBC collection that could decrease bias related to EBC sampling and H2O2 and MDA measurement.

This study provides original data on ways of H2O2 and MDA exhalation in healthy subjects and also has sound methodological conclusions. They can be useful in future investigations devoted to non-invasive monitoring of inflammation and/or per-oxidative processes in the airways.

Author’s reply: We thank the reviewer for taking time to make recommendations aimed at improving our manuscript. The positive comments have been noted and appreciated. In the revised version, we have done our best to address and clarify each of the points raised.

Major Compulsory Revisions

What was the duration of the clinical part of study (EBC collection from 24 subjects)? If this exceeded 3-4 months the possible influence of climate changes on EBC parameters should be discussed.

Author’s reply: The duration of the clinical part of study was about 2 weeks in February, thus avoiding the possible influence of climate changes on EBC parameters. The sentence “The duration of the clinical part of the study was about 2 weeks, thus avoiding the possible influence of climate changes on EBC parameters” has been added in the “Methods” section (page 5 on “Study design” sub-paragraph).

Did author perform the control experiments i.e. four consecutive EBC collecting sessions at the same cooling temperature? The results of this experiment performed with e.g. 4-5 subjects for each cooling temperature should be described in the results section to provide data on baseline inter-sessions variability of EBC parameters.

Author’s reply: This is a very important issue still poor discussed in the EBC collection. Further studies are in progress with a larger number of subjects to perform a complete reliability test on different EBC parameters, calculating Cronbach Alpha and intra-class correlation coefficient. In this case, 10 out of 24 controls enrolled for this study performed four consecutive EBC collection at the two extremes of temperature (-10 °C and +5 °C). We measured EBC total volume, conductivity and H2O2, whereas MDA reliability has been previously discussed (Rapid Commun Mass Spectrom. 2003;17(7):637-45, Eur Respir J. 2004 Dec;24(6):1011-7). The sentence: “To assess inter-sessions variability, 10 out of 24...”
subjects performed four consecutive EBC collection at the two extremes of temperature (-10 °C and +5 °C)” has been added “Methods” section (page 5 on “Study design” sub-paragraph). No significant differences were found among times, as shown in the new table 2 of the revised manuscript (page 24), and this statement has been added in “results” section (page 8). Then, the following sentence was introduced in the discussion section (page 12) “Intra-sessions variability study (table 2) showed that observed differences between markers collected at different temperature can not be ascribed to intra-individual variability. MDA reliability was not assessed, because it had already been demonstrated [15,16]”. A reference (ref. 16) has been added.

Discussion (Page 13) “H2O2 and MDA were positively correlated… These data suggest … biological induction of hydrogen peroxide (a marker of inflammatory processes) could lead to enhance of in vivo oxidative stress, with increase in lipid peroxidation products.” This is a little too strong and not supported by obtained results. Study subjects were without any inflammatory processes especially in the respiratory tract.

Author’s reply: We do agree with the reviewer. we changed the statement into: “hydrogen peroxide production and lipid peroxidation could be related processes in vivo also in healthy subjects, as already observed in subjects with inflammatory processes of the respiratory tract”.

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

Was the collecting device equipped with flow meter? Authors mentioned (in “Results”) that there was the significant correlation between volume of exhaled air and condensed volume.

Author’s reply: The sentence we wrote in the “results” section refers to previously data from our lab. TURBO DECCS may be equipped with an ultrasonic flow meter, which can be placed close to the mouthpiece. The flow meter permits the counting of exhaled air without filtering exhaled breath. Preliminary data showed that there is a strong positive correlation between exhaled air volume and total EBC volume, as shown n the figure)

![](image)

Discretionary Revisions (which the author can choose to ignore)
Unable to decide on acceptance or rejection until the authors have responded to the What next?: e major compulsory revisions
Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable
Statistical review: No
Declaration of competing interests:
I declare that I have no competing interests.
Reviewer's report
Influence of condensation temperature on selected exhaled breath parameter
Title: 
Version: 1 Date: 16 June 2005
Reviewer: Stelios Loukides
Reviewer's report:

General
This is a study on the effect of condensation temperature on parameters of exhaled breath condensate and levels of selected biomarkers in healthy subjects. The main limitation of this study is whether the observed changes in different cooling temperatures can be attributed to the temperature or express the absence of repeatability in these biomarkers within the four measurements. Additionally, the term "control subjects" is based on clinical history and not on subjective measurements like BHR and atopy status.

Author’s reply: We thank the reviewer for taking time to make recommendations aimed at improving our manuscript. In the revised version, we have done our best to address and clarify each of the points raised.

Major Compulsory Revisions
1. The main limitation of their methodology is whether the observed changes in different cooling temperatures can be attributed to the temperature or express the absence of repeatability in these biomarkers within the four measurements. I believe that trying to come to conclusions from their data without checking repeatability leads to weaker conclusions for the cooling temperature.

Author’s reply: This is a very important issue still poorly discussed in the EBC collection. Further studies are in progress with a larger number of subjects to perform a complete reliability test on different EBC parameters, calculating Cronbach Alpha and intra-class correlation coefficient. In this case, 10 out of 24 controls enrolled for this study performed four consecutive EBC collection at the two extremes of temperature (-10 °C and +5 °C). We measured EBC total volume, conductivity and H2O2, whereas MDA reliability has been previously discussed (Rapid Commun Mass Spectrom. 2003;17(7):637-45, Eur Respir J. 2004 Dec;24(6):1011-7). The sentence: “To assess inter-sessions variability, 10 out of 24 subjects performed four consecutive EBC collection at the two extremes of temperature (-10 °C and +5 °C)” has been added “Methods” section (page 5 on “Study design” sub-paragraph). No significant differences were found among times, as shown in the new table 2 of the revised manuscript (page 24), and this statement has been added in “results” section (page 8). Then, the following sentence was introduced in the discussion section (page 12) “Intra-sessions variability study (table 2) showed that observed differences between markers collected at different temperature cannot be ascribed to intra-individual variability. MDA reliability was not assessed, because it had already been demonstrated [15,16]”. A reference (ref. 16) has been added.

2. Subjects were characterized as controls using a brief clinical history and spirometry. No subjective measurements like BHR and atopy were used. These measurements might be useful for younger ages since they provide proven data for the term control. Authors need to clarify this limitation even in a separate paragraph in the discussion.

Authors’ reply: We agree with the reviewer that a better subject characterization may be useful for a proper data interpretation. For this study, we recruited volunteers belonging to University staff of our
laboratory; this group served as control group in previous studies and therefore they have already been evaluated in terms of atopy and BHR, both negative.

In the revised manuscript we wrote: “Healthy subjects were defined as asymptomatic and non atopic individuals, with normal spirometry and negative bronchial hyper-responsiveness to methacholine.” (page 5 in the “methods” section, “Subjects” sub-paragraph).

3. Did subjects wear a nose clip?

Authors’ reply: we added, as requested: “without nose clip” (page 6). In this study, we decided to make EBC collection without a nose clip, because it was difficult to use over the course of 40 minutes. In addition, it is still uncertain whether nose clip drainage into the mouth from the nose, thus interfering exhaled mediator levels.

4. EBC collection in this study was performed with a transportable collector. Authors have to make clear if some subjects collected samples at home. If yes then they have to discuss if these measurements might affect their data.

Authors’ reply: The subjects collected their EBC all in the same laboratory and no one collected EBC at home. This statement has been added in the “methods” section, “EBC collection” sub-paragraph (page 6).

5. Authors must clarify the period of storage. Which was the interval between measurements? Why did they not check stability? Analysis of hydrogen peroxide was performed within 2-3 days after EBC collection. Did the same happen with malondialdehyde?

Authors’ reply: All the subjects collected their EBC in 3 consecutive weeks and in the same section all the four samples of the same subject were collected and stored within one hour. Conductivity did not present any stability problem, because a contamination of storage vial to the EBC and its non volatile molecules content were excluded repeating different measures of conductivity in pure water kept in the vials for different periods of time. We prefer to detect hydrogen peroxide in EBC within 2-3 days to avoid degradation problems due to its relative instability (stability up to a week, see Nowak et al, Free Rad Biol Med, 2001). The stability of MDA is higher (up to 8 months), as already shown in a previous work (Eur Respir J. 2004 Dec;24(6):1011-7). However, MDA was measured within 2 weeks after -80 °C freezing. These statements have been added in the “methods” section, “EBC-analysis” sub-paragraph (pages 6-7). The references (14 and 16) have been added.

6. I did not understand the significance of correlation data between biomarkers and conductivity. This issue must be further discussed by the authors.

Authors’ reply: If the number of biomarker molecules depended only from droplets present in EBC and the biomarker evaporation was negligible, so, with healthy subjects where pathological processes do not affect the composition of airway lining fluid, biomarker concentrations and conductivity should be correlated with a relatively high Pearson’s r, because their concentrations would be strictly related to the number of exhaled lining fluid droplets. In fact, Effros et al., J. Appl. Physiol (2005) showed that different parameters measured in EBC (salts, conductivity, urea), which should depend only by droplets EBC content, were correlated with each other. The lack of correlation between a condensed biomarker and conductivity clearly shows that droplets are not enough to determine its content in EBC, and other
contributing factors should operate. In the “discussion” section (page 13-14), we wrote:” this suggests that a slight contribution to the total concentration of MDA in EBC could derive from MDA-containing droplets. In fact, the lack of correlation between conductivity and volatile components indicates that non-volatile ions reflect the number of airway lining fluid droplets joining the vapor stream, thus accounting for a mechanism complementary to evaporation. The latter seems to be the main determinant for the EBC content of H₂O₂ (which is volatile), and MDA (which is also slightly volatile).”

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Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

Discretionary Revisions (which the author can choose to ignore)

**What next?:** Unable to decide on acceptance or rejection until the authors have responded to the major compulsory revisions
An article whose findings are important to those with closely related research **Level of interest:** h

**Quality of written English:** Acceptable

**Statistical review:** No

**Declaration of competing interests:**
'I declare that I have no competing interests'