**Author's response to reviews**

**Title:** Inhaled beta-agonist does not modify sympathetic activity in patients with COPD

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**Author's response to reviews:** see over
Dear Editor,

Please find enclosed our revised manuscript entitled: “Inhaled β-agonist does not modify sympathetic activity in patients with COPD”.

We would like to thank the referees for their competent and fair review of our manuscript. Taking up all the points and suggestions was very informative for us and we would like to thank you for your most valuable support. I have added a point-by-point reply responding to the questions raised. We hope that the revised version of our manuscript will meet all the requirements necessary to enable its publication. It was read and approved by all authors.

Sincerely yours,

Helge Haarmann, MD
Point-by-point reply

We would like to thank both reviewers for their competent and fair review of our manuscript, which helped us to make substantive improvements to our work.

In the following, we reply to each of the comments.

Reviewer 1: Arnoldus J. R. van Gestel

Comment 1 & 2 (Abstract):

Information about the variables (HRV, BRS) and the methods used (Finapres) are missing. Also the duration of the assessment is missing.

The results should include the #2-agonists induced changes/ differences of all the main variables (HR, HRV, BRS).

Reply:

We added the following information to the methods section of the abstract (line 44):

“MSNA, heart rate, blood pressure, and respiration were continually measured. After baseline recording of 20 minutes, placebo was administered; after further 45 minutes salmeterol (50 μg) was administered which was followed by a further 45 minutes of data recording. Additionally, lung function, plasma catecholamine levels, arterial pulse wave velocity, heart rate variability, and baroreflex sensitivity were evaluated.”

Our primary endpoint is the effect of salmeterol on MSNA. We evaluated other parameters as secondary endpoints, but we believe, the results section of the abstract should focus mainly on the primary endpoint. Otherwise it could be difficult for the reader to grasp the key message.

We added the following results (line 55):

“Heart rate increased significantly by 3.8 ± 4.2 (p<0.01) acutely and 3.9 ± 4.3 bpm (p<0.01) after 4 weeks.”

Comment 3 (Background):

The authors formulated their aim of the study as followed: « We aimed to investigate... » I would recommend writing it in a more neutral form like: This study investigates the effect....

Reply:

The proposed change was accepted (line 91).
Comment 4 (Background):

The association between sympathetic tone and chemoreflexes, baroreflexes and lung hyperinflation needs to be more elucidated. The abnormality of autonomic function in patients with COPD may affect stimulus reception, afferent nerve conduction, central processing, efferent nerve conduction, and neuromuscular response. The sensory receptors that might play a significant role in autonomic dysfunction in patients with COPD are arterial and cardiac baroreceptors, metabolic and pulmonary stretch receptors, bronchopulmonary C-fibres and arterial chemoreceptors. Large intrathoracic pressure changes, as occurring in chronic obstructive pulmonary disease due to hyperinflation, are transmitted to the heart and great vessels and can influence both peripheral baroreceptors and cardiac performance. Large pressure changes may cause fluctuations in cardiac performance, and therefore, in systemic blood pressure provoking finely modulated compensatory changes of the heart rate mediated by separate outputs of intrathoracic baroreceptors.

Reply:

Our study was not designed to unravel the complex mechanisms causing increased sympathetic activation in COPD. However, we now cite a review of Arnoldus van Gestel elucidating this topic (line 82, reference no. 19).

Comment 5 (Methods):

The authors included subjects with COPD GOLD stage II or III. Why were COPD GOLD stage IV exclude from the study?

Reply: Patients in GOLD stage IV are more likely to be unstable. Thus a one-month protocol needing stable medication would have been demanding. Furthermore the necessary abstention of effective inhaled therapy at the beginning of the protocol might have been a problem in severely diseased patients.

Comment 6 (Measurement and data analysis):

« The 2-hour measurement period comprised the continuous recording of MSNA, respiration and other measurements as explained below (...) » (122). Please describe all measurements because it is very uncommon to refer to paragraphs who are yet to come.

In addition « MSNA and the other following parameters were only assessed (...) » (132) Here it is not clear which following parameters are meant to be assessed. Please explain more precisely what is meant.

Reply:

We now list all parameters that were continuously recorded (line 127):

“The 2-hour measurement period comprised the continuous recording of MSNA, ECG, blood pressure, respiration, oxygen saturation, and transcutaneous CO2 with the patient lying in a supine position.”
We changed the following sentence for more clarity:

Old:

“MSNA and the other following parameters were only assessed during the final 10 minutes of each of these sections (baseline, placebo, salmeterol).”

New (line 132):

“We analyzed the last 10 minutes of each of these recording sections (baseline, placebo, salmeterol).”

Comment 7 (Measurement and data analysis):

„Under the assumption that adequate microneurographic recordings can only be obtained in 75% of all subjects studied (...). Where does this assumption come from? Please underline this statement with literature.

Reply: We comment on success rates in the discussion section, where we also added a reference. We refer to our reply to Comment 1 of Reviewer Surya Bhatt.

We changed the following sentence (line 163):

“Under the assumption that adequate microneurographic recordings would only be obtained in 75% of all subjects studied in our lab, 32 subjects needed to be enrolled.”

Comment 8 (Results):

In the result part there is a good division of the individual parameter assessed. It would be helpful to see this division already in the method section (cardiovascular, respiratory and autonomic nervous system). Further a graphical overview of the assessed parameters would be helpful.

Reply:

We slightly changed the structure and added the following headlines to improve the methods section.

Study protocol (line 123)

Muscle sympathetic nerve activity (line 134)

Hemodynamics, baroreflex sensitivity, and heart rate variability (line 141)

Catecholamine and BNP measurements (line 145)

Lung function, pulse wave velocity, and echocardiography (line 149)

Old:
**Muscle sympathetic nerve activity**

Sympathetic tone was measured using microneurographic recordings of efferent muscle sympathetic nerve activity in the peroneal nerve[21]. A tungsten microelectrode (shaft diameter 200 µm, tip diameter 1-5 µm, FHC, Bowdoin, USA) was inserted into the nerve. The signal was amplified and integrated (662C-4 Nerve Traffic Analysis System, Absolute Design and Manufacturing Services, Iowa, USA). Intraobserver variation in identifying bursts of efferent sympathetic nerve activity was ~5% in previous studies done in our laboratory[15, 22].

About 20 minutes after a stable MSNA signal had been obtained ('baseline'), one dose of placebo was administered; after a further recording period of 45 minutes one dose of salmeterol (50 μg) was administered which was followed by a further 45 minutes of data recording. MSNA and the other following parameters were only assessed during the final 10 minutes of each of these sections (baseline, placebo, salmeterol).

**Comment 9 (Results):**

Baseline MSNA was elevated in all patients. Please include normal values incl. references to the literature.

**Reply:** We now added a reference to historical values of healthy subjects of similar age and BMI that were obtained in a former study of our lab.

**New (Line 194):**

“Baseline MSNA was 74.4 ± 16.3 bursts/100 heart beats. This was higher as compared to historical controls[26] of similar age and BMI.”
Comment 10 (Results):

A valid signal for visit 1 was obtained in 18 patients, while in 14 patients a valid signal could not be obtained. How do the authors explain this fact?

Reply:

We comment on success rates of MSNA recordings in the discussion section. We refer to our reply to Comment 1 of reviewer Surya Bhatt.

Comment 11 (Results):

The layout/ format of the tables is inadequate. It is unable to get a clear overview of the data. Furthermore there are a lot small errors (p=>0.01, P= 0.01, p= 0.01). It looks like there are p-values missing for lung function parameters.

Reply:

We changed the style of the tables for better readability and corrected the mentioned errors.

Comment 12 (Discussion):

The discussion is poorly structured. I would suggest that the second paragraph (effects on heart rate) underlines the short term effects of B2 agonist on heart rate en heart rate variability. There is clear evidence that B2 agonist increases sympathetic tone and decreases parasympathetic tone. In addition the long term effects can be discussed.

Reply:

We structured the discussion in a way that we focus on the effects of heart rate first, as we believe heart rate is of great interest to the reader. Then we discuss the effects of salmeterol on sympathetic nerve activity.

Please note that changes in HRV as a measure of autonomic nervous system activity are difficult to interpret, especially if heart rate changes (reference no. 41). This is one reason why we used microneurography to assess sympathetic activity.

For discussion of effects of beta-2-agonists on MSNA see line 255-269.

Comment 13 (Discussion):

“The fact that salmeterol......autonomic nervous system” (226). Is this really true?

Reply:

We now phrased this statement less definite.
The fact that salmeterol treatment was associated with an increase in heart rate without an increase in MSNA in our study indicates that the increase in heart rate is caused by activation of cardiac β-receptors rather than efferent excitatory traffic of the autonomic nervous system.

The fact that salmeterol treatment was associated with an increase in heart rate without an increase in MSNA in our study suggests that the increase in heart rate is caused by activation of cardiac β-receptors rather than efferent excitatory traffic of the autonomic nervous system.

**Comment 14 (Discussion):**

I would suggest that the third paragraph (sympathetic activation) underlines the short term effects of B2 agonist on MSNA. In addition the long term effects can be discussed.

**Reply:**

We especially discussed the short term and long term effects in line 288-294.

**Comment 15 (Discussion):**

In an additional paragraph the contradictorily results of HR/HRV versus MSNA can be discussed.

**Reply:**

We shortly discussed the results of HRV and MSNA in line 270-274. Our focus is on the effects of salmeterol on MSNA as the primary endpoint, therefore we did not further expand the discussion on HRV data due to word limitations.

**Comment 16 (Other details):**

Please make sure that the unnecessary line numbers are deleted in the whole manuscript. Just write line numbers where there is some text. Further it would be nice to shorten the whole manuscript.

**Reply:**

The layout of the manuscript resides with the journal editorial office. Line numbers will not be published.

**Comment 17 (Other details):**

Please make sure there are no blank pages in the manuscript (page 19)

**Reply:** We removed the blank page.
Comment 18 (Other details):

Table 1 is not placed in the centre of the page. Please make sure to put the legends on the same page as the table itself. Table 2 and 3 should be placed on one page rather than split off two pages. Further the first column is very confusing in all tables. It would be good to put some extra work in the layouts of the tables to get them look more attractive.

Reply: The layout of the manuscript resides with the journal editorial office.

Comment 19 (Other details):

Table 2,3 and 4 are very confusing because there are different n postulated between baseline and endpoint measure. Change between different n is not meaningful. Please include just participants which completed measurements at baseline and after salmeterol.

In Table 4 there are problems in data presentation:

- Inconsistent decimal places (column 1)
- Layout Mean/ SD not appropriate
- Title of table 4 is not appropriate

Reply:

- For better readability, we now removed the n values of tables 2-4.
- The last value in column 1 of Table 4 would be -0.00; that is why we had to add another decimal place (0.002).
- We corrected the title of Table 4:

Old:

Capillary blood gas analysis at baseline.

New:

Capillary blood gas analysis.

Comment 20 (Other details):

The graphs on Page 28 and 29 are unsharp printed and don’t look attractive at all. See comment 16. Make sure to put the Figure legends from page 27 to the figures appropriate.

Figure 1: Why did you use bar chart to illustrate this result? I would recommend a trend figure.

Reply:
The print quality of the figures resides with the journal. We believe a trend line in Figure 1 might not be appropriate as it suggests a time trend which is not exactly what is displayed.

Comment 21 (Other details):

The layout of the graphs a not attractive... They need further styling. Do we need figure 2 as there are only 12 dots?

Reply:

The scatter plot is of interest since it links norepinephrine data (which are known to most readers) to MSNA data.
Reviewer 2: Surya Bhatt

Comment 1 (Major):

There was a significant reduction in the actual number of subjects studied, significantly decreasing the power of the study. This needs more comment.

Reply:

We further extended our comment on MSNA success rates in the discussion.

Old:

“The calculated sample size of 24 patients for the primary endpoint was missed by 6 subjects (n=18), as the failure rate of MSNA registration was higher than expected. As a consequence, we were unable to detect a significant difference in MSNA changes between placebo and salmeterol of the magnitude we were expecting. The difficulty to keep a stable MSNA signal for a data registration period of almost two hours was underestimated, as previous studies[15, 16] had distinct shorter registration periods.”

New (line 300-305):

“The calculated sample size of 24 patients for the primary endpoint was missed by 6 subjects (n=18), as we overestimated the rate of successful MSNA registrations. The per visit success rate of MSNA recordings is reported as 70% in literature[46]. We had a 56 % success rate on visit 1. The difficulty to keep a stable MSNA signal for a data registration period of almost two hours was underestimated, as previous studies in our lab had distinct shorter registration periods[15, 16]. As a consequence, the power of the study was slightly decreased.”

Comment 2 (Major):

It is known that an increase in resting heart rates is associated with an increase in mortality. While the authors report a 4 BPM increase in HR acutely after salmeterol administration at both visits, what was the impact on resting heart rate?

Reply:

We added this information to the results section (line 206):

“Resting heart rate after 4 weeks of salmeterol treatment increased from 65.6 ± 9.4 at baseline to 67.1 ± 9.9 bpm at visit 2 (before the administration of salmeterol; p=0.04 in paired t-test).

Comment 3 (Major):

The administration of salmeterol for 4 weeks could potentially result in saturation of beta receptors, thus preventing further effects at visit 2. The authors should comment on this.
We studied not only the acute but also the long-term effects of salmeterol over one month. As in the acute setting, MSNA and other measures of autonomic nervous system activity were unchanged. Desensitization or saturation of β2-adrenoceptors that follows regular use of β2-agonist treatment is believed to be responsible for the resolution of hemodynamic findings after the first days and weeks and may also prevent long-term effects of salmeterol on MSNA [5]. However, in our study the acute effects were comparable to the effects after 4 weeks. Specifically the increase in heart rate was similar in the acute and long-term setting.

Comment 4 (Major):

Half the patients were current smokers. Smoking is known to increase sympathetic activation. Was this controlled for?

Reply:

Patients had been instructed not to smoke any cigarettes for 8 hours before the visit, to rule out possible acute effects of smoking on MSNA. We now included the comparison between smokers and non-smokers regarding baseline. No effect of smoking status on MSNA was detected.

Chronic effects of smoking on MSNA are not clearly established. We just have submitted a study with 85 subjects investigating this relation without clear-cut results.

New (line 195):

“No significant effect of smoking status on baseline MSNA was observed (smokers: 75.91±15.44 vs. non-smokers: 71.58±15.20 bursts/100 heart beats; p= 0.54).

Comment 1 (Minor):

The authors state that the baseline MSNA was elevated. Please provide reference values for normal subjects.

Reply:

We refer to our reply to comment 9 of reviewer Arnoldus van Gestel.

Comment 2 (Minor):

Results.. Lines 194 and 195..“As an acute effect of salmeterol, low frequency power (reflecting sympathetic tone) was significantly elevated while high frequency power (reflecting parasympathetic tone) decreased”. Please clarify what measurements these results pertain to.
Reply:

We now added the parameters.

Old:

“As an acute effect of salmeterol, low frequency power (reflecting sympathetic tone) was significantly elevated while high frequency power (reflecting parasympathetic tone) decreased.”

New (line 208):

“As an acute effect of salmeterol, low frequency power (LF) of HRV, reflecting sympathetic tone, was significantly elevated while high frequency power (HF), reflecting parasympathetic tone, decreased.”