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COPD patients with peripheral airway obstruction reversibility identified by exhaled nitric oxide

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**Abbreviations:**

- **BDP**: beclomethasone dipropionate
- **FENO**: fractional exhaled nitric oxide concentration
- **FEV₁**: forced expired volume in 1 second
- **FVC**: forced vital capacity
- **FRC**: functional residual capacity
- **He**: helium
- **ICS**: inhaled corticosteroids
- **LABA**: long-acting β₂-agonist
- **LAMA**: long-acting muscarinic antagonist
- **NO**: nitric oxide
- **ppb**: part per billion
- **S**: slope of phase III of the single-breath washout test
- **SF₆**: sulfur hexafluoride
- **SpO₂**: peripheral capillary oxygen saturation
Abstract (243 words)

**Rationale:** Besides its role as an inflammatory marker in asthma, fractional exhaled nitric oxide (FeNO) provides information on the extent of the airway obstruction process through evaluating its change after bronchodilation.

**Objective:** To investigate whether FeNO change after bronchodilation can identify different sites of airway obstruction in COPD patients.

**Methods:** FeNO, FEV₁ and the slopes (S) of the alveolar plateau of the single breath washout test (SBWT) were measured in 61 stable COPD patients (FEV₁ < 34.5% predicted) before and after the inhalation of 400 µg salbutamol. SBWT used Helium (He) and sulfur-hexafluoride (SF₆). Obstruction relief occurring in pre-acinar and intra-acinar small airways is expected to decrease Sₜₐₜ and Sₜₐₙ, respectively. Indices changes (Δ) after bronchodilation were expressed as a percentage of pre-bronchodilation values.

**Results:** FeNO stability (|ΔFeNO|≤11%) was observed in 19 patients [-2.7(6.7)%] [mean (SD)] (NO= group); ΔFeNO>11% [+37.4(27.7)%] in 20 patients (NO+ group) and ΔFeNO<-11% in 22 patients [-31.2(9.8)%] (NO- group). A similar ΔFEV₁ (p=0.583; [+9.4(9.6)%]) was found in the three groups. In NO= and NO+ groups, neither Sₜₐₜ nor Sₜₐₙ changed; in NO- both Sₜₐₜ [-12.4(27.5)%, p=0.007] and Sₜₐₙ [-20.2(20.4)%, p<0.001] significantly decreased.

**Conclusion:** Different patterns of FeNO response to β₂-agonists were observed in COPD most likely depending on the extent of the dilation process. A profile of airway obstruction with an extensive β₂-agonist response down to lung periphery is identified by FeNO reduction after acute bronchodilation in 30% of COPD patients. The clinical relevance of this profile requires further investigation.
Introduction

Fraction of exhaled nitric oxide (F\textsubscript{E}NO) has been extensively investigated in asthma (1) and is frequently used to monitor type-2 airway inflammation in asthma management (2). Conversely, a role for F\textsubscript{E}NO in chronic obstructive pulmonary disease (COPD) management has not been clearly identified so far (3) even if a recent meta-analysis did show a slight increase of F\textsubscript{E}NO in patients with COPD compared to healthy controls (4). In fact, F\textsubscript{E}NO appears to be elevated, only in some phenotype of COPD associated with airway eosinophilia (5-8), a certain degree of reversibility (6) and an increased risk of exacerbation (9). The eosinophilic COPD patients are likely to better respond to corticosteroids (10-12). In any case, the benefit of F\textsubscript{E}NO-guided treatment was, so far, rather modest (13, 14) or inexistent (15) in COPD patients. In addition, the well-known interference of active smoking with F\textsubscript{E}NO levels (16) makes it more difficult to define relevant cut-offs (17). Together, all this explains why F\textsubscript{E}NO measurement is not recommended in the management of COPD (3).

However, apart from its usual function as a type-2 inflammatory biomarker in asthma, F\textsubscript{E}NO was also shown to be helpful in revealing different patterns of airway obstruction that were not apparent based on FEV\textsubscript{1} (18). Briefly, F\textsubscript{E}NO, being sensitive to airway calibre reduction induced by airway challenge (19-21), was surprisingly shown to be relatively stable after acute bronchodilation (22, 23) even though one study has shown a mild F\textsubscript{E}NO increase in this condition (24). In fact, using a single breath wash-out test (SBWT), we were able to demonstrate that this global result actually masked three different behaviours of F\textsubscript{E}NO response to acute bronchodilation: a decrease likely due to the relief of an airway obstruction up to intra-acinar airways leading to an amplification of the NO back-diffusion (25, 26), an increase likely associated with a predominant dilation up to pre-acinar airways, and a F\textsubscript{E}NO stability when obstruction relief involved predominantly central airways (18).
In COPD, both FENO stability (27, 28), rather modest FENO increase (29) or decrease (30) were described after bronchodilation. However the FEV₁ changes in these studies were far less impressive than those observed in asthma.

With the above in mind, the aim of this study was to evaluate whether this apparent absence of effect might also mask divergent patterns of FENO responses to acute bronchodilation that would also allow to identify different profiles of airway obstruction in COPD patients.

To this end, single breath washout tests (SBWT) were performed in order to reflect the location of the airway obstruction sites (31, 32).

Methods

Patient population

Patients with documented COPD referred to the outpatient COPD clinic of the Chest Department in the Erasme University Hospital were considered for recruitment from February to September 2016. At baseline, functional dyspnoea using the modified Medical Research Council (mMRC) scale and the number of exacerbations over the previous 12 months were recorded (based on the medical file and the anamnesis performed during the clinical visit). Moderate exacerbations were defined as requiring treatment with systemic corticosteroids or antibiotics and severe exacerbations requiring hospitalization or evaluation in the emergency department (33). Participants were not permitted to take short-acting β₂-agonists and muscarinic antagonists for at least 6 hours and long-acting β₂-agonists (LABA), muscarinic antagonists (LAMA) and inhaled corticosteroids (ICS) for at least 48 hours.

Before the study started, the protocol was approved by the local ethics committee (B406201526529) of the Erasme University Hospital on February 12th 2016 and all participants provided written informed consent.
Inclusion criteria were a diagnosis of COPD according to internationally established criteria (post-bronchodilator FEV₁/FVC less than 70%) (3); aged 40 years or older; smoking history equivalent to at least 10 pack-years; and stable medical therapy.

Exclusion criteria were a diagnosis of asthma, asthma-COPD overlap (ACO) based on clinical consensus among members of our asthma-COPD clinics, using the GOLD-GINA for the diagnosis of diseases of chronic airflow limitation (34) or another respiratory disease which could influence the results of the study; a respiratory infection or COPD exacerbation over the previous 6 weeks; an inability to perform acceptable tests; and an inability to maintain the washout period.

**Study design**

This was a prospective and open study.

FE(NO) values, spirometry and single-breath washout tests (SBWT) were sequentially performed before and 20 minutes after the inhalation of 400µg of salbutamol administered via a spacer device.

**Assessments**

(a) **Spirometry**

A spirometry (Zan®, Oberthulba, Germany) and a diffusing capacity for carbon monoxide (DL<sub>CO</sub>) were conducted according to the standard guidelines for pulmonary function testing (35). The DL<sub>CO</sub> was assessed by the single breath method (36). Forced expiratory volume in 1 second (FEV₁), forced vital capacity (FVC), and DL<sub>CO</sub> were expressed as a percentage of the predicted normal values (37).

(b) **Single-breath washout test (SBWT)**

SBWT was used to assess ventilation distribution. This test consists in one litre inhalation from functional residual capacity (FRC) followed by an expiration down to residual volume (RV),
both at a constant flow (0.4 L/s). The inhaled mixture is composed of 5% Helium (He) and 5% sulfur hexafluoride (SF\textsubscript{6}) in oxygen (O\textsubscript{2}).

Expired gas concentrations were measured by a quadruple mass spectrometer (LR6000 Logan-Sinclair, Rochester, UK). The slopes for each inert non-resident gas (S\textsubscript{He} and S\textsubscript{SF\textsubscript{6}}) were evaluated by a linear fitting on the phase III (alveolar plateau) of the concentration vs expired volume curves. Tests were performed in order to give three slope values with a variation coefficient not exceeding 10%. The mean value was considered as the final value. Additional information on this technique is described in Michils \textit{et al} (38).

\textit{(a) Fractional exhaled nitric oxide (F\textsubscript{E}NO)}

F\textsubscript{E}NO values were measured according to the international standards (1), before any spirometry, using a LR 2000 chemiluminescence analyser (Logan Research LTD, Rochester, UK) at a flow rate of 50ml.s\textsuperscript{-1}. These values were expressed in parts per billion (ppb).

A value of 11\% change was previously reported to be the short-term variability of FENO measured in COPD patients by chemiluminiscence (39). Therefore, this value was chosen as a threshold to define a significant F\textsubscript{E}NO change after the \textbeta\textsubscript{2}-agonist action.

\textbf{Statistical methods}

\textit{(a) Baseline values}

F\textsubscript{E}NO, FEV\textsubscript{1}, FVC, S\textsubscript{He}, and S\textsubscript{SF\textsubscript{6}} baseline differences among the groups were evaluated by an ANOVA analysis, F\textsubscript{E}NO was log-transformed.

\textit{(b) F\textsubscript{E}NO and lung function changes}

The bronchodilation effect in each group was assessed by a linear mixed model analysis. The fixed effects were the bronchodilation status and the group identifier whereas the random effect
of inter-subject variability on pre-bronchodilation values (random intercept model) was considered. The R software (version 3.3.2) (40) was used. A p-value less than 0.05 (bilateral) was regarded as a significant difference.

**Results**

(a) **Baseline characteristics**

Sixty-one COPD participants were recruited. Considering a FeNO change of 11% as significant after bronchodilation (39), three groups were identified (Figure 1): 19 participants (31%) did not change their FeNO by more than 11% of baseline (NO= group), 20 participants (33%) increased their FeNO by more than 11% (NO+ group) and 22 participants (36%) decreased their FeNO by more than 11% (NO- group).

**Table 1.** Participants characteristics, FeNO and baseline lung function.

<table>
<thead>
<tr>
<th></th>
<th>Total (n=61)</th>
<th>NO= group (n=19)</th>
<th>NO+ group (n=20)</th>
<th>NO- group (n=22)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>63 ± 10</td>
<td>64 ± 7</td>
<td>64 ± 11</td>
<td>61 ± 11</td>
<td>0.558</td>
</tr>
<tr>
<td>Gender (F/M)</td>
<td>25/36</td>
<td>8/11</td>
<td>6/14</td>
<td>11/11</td>
<td>0.393</td>
</tr>
<tr>
<td>Current smokers (%)</td>
<td>39</td>
<td>58</td>
<td>30</td>
<td>32</td>
<td>0.136</td>
</tr>
<tr>
<td>Pack-years</td>
<td>51.4 ± 23</td>
<td>56.1 ± 19.3</td>
<td>55.6 ± 29.5</td>
<td>41.8 ± 14.5</td>
<td>0.114</td>
</tr>
<tr>
<td>mMRC score</td>
<td>2.2 ± 0.9</td>
<td>2.2 ± 1.1</td>
<td>2.1 ± 0.8</td>
<td>2.3 ± 0.8</td>
<td>0.785</td>
</tr>
<tr>
<td>Exacerbations in the</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>previous year (%)</td>
<td>28/10/62</td>
<td>32/10/58</td>
<td>20/15/65</td>
<td>31/5/64</td>
<td>0.753</td>
</tr>
<tr>
<td>(0/1/2 or ≥1 requiring</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hospitalisation)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GOLD grade (1/2/3/4) (%)</td>
<td>10/41/29/20</td>
<td>16/21/42/21</td>
<td>5/65/15/15</td>
<td>9/36/32/23</td>
<td>0.182</td>
</tr>
<tr>
<td>GOLD group (A/B/C/D) (%)</td>
<td>13/29/5/53</td>
<td>17/28/5/50</td>
<td>10/30/10/50</td>
<td>14/27/0/59</td>
<td>0.895</td>
</tr>
<tr>
<td>FeNO (ppb)</td>
<td>10.4 [5.3 - 20.6]</td>
<td>10.9 [6.4 - 18.4]</td>
<td>8.2 [3.9 - 17.4]</td>
<td>12.4 [6.2 - 24.8]</td>
<td>0.143</td>
</tr>
<tr>
<td>FEV1 (%pred)</td>
<td>34.5 ± 18.3</td>
<td>32.9 ± 21.7</td>
<td>35.3 ± 14.5</td>
<td>35 ± 19.1</td>
<td>0.911</td>
</tr>
</tbody>
</table>
The three groups had no significant differences in baseline characteristics; they had a similar severe degree of airway obstruction, a similar severe reduction of diffusing capacity, similar SpO2 and FeNO levels (Table 1) and a similar ongoing pharmacological treatment including a similar dose of inhaled corticosteroids (Table 2). No differences were observed in the symptoms, the number of exacerbations in the previous year and the severity of COPD, showing a similar proportion of GOLD groups (A, B, C, D) and grades (1, 2, 3, 4) in the three NO groups (Table 1).

Table 2. Pharmacological treatment characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Total (n=61)</th>
<th>NO= group (n=19)</th>
<th>NO+ group (n=20)</th>
<th>NO- group (n=22)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>LABA (% of patients)</td>
<td>84</td>
<td>79</td>
<td>85</td>
<td>86</td>
<td>0.830</td>
</tr>
<tr>
<td>LAMA (% of patients)</td>
<td>79</td>
<td>74</td>
<td>80</td>
<td>82</td>
<td>0.858</td>
</tr>
<tr>
<td>ICS (% of patients)</td>
<td>49</td>
<td>42</td>
<td>45</td>
<td>59</td>
<td>0.531</td>
</tr>
<tr>
<td>ICS dose (µg BDP eq day⁻¹)</td>
<td>1083 ± 510</td>
<td>1063 ± 417</td>
<td>1111 ± 546</td>
<td>1077 ± 572</td>
<td>0.965</td>
</tr>
</tbody>
</table>

Data are presented as percentage (% ) of patients and mean ± standard deviation for the ICS dose; p is the statistical significance of group effect in an exact Fisher test except for ICS dose (one-way ANOVA). LABA: long-acting β₂-agonists; LAMA: long-acting muscarinic antagonists; ICS: inhaled corticosteroids; BDP: beclomethasone dipropionate.

(b) Changes in FeNO values and lung function parameters (mean (SD))

After bronchodilation, all the COPD participants and the NO= group showed a non-significant change of FeNO (-0.8±4.8 ppb (0.2±33.4% of baseline), p =0.124 and -0.4±0.9 ppb (-2.7±6.7%
of baseline), p=0.243 respectively); whereas in the NO+ group, a significant increase of 3.1±2.4 ppb (37.4±27.7% of baseline) (p< 0.001) and the NO- group, a significant decrease of -4.8±3.3 ppb (-31.2±9.8% of baseline) (p<0.001) were recorded (Fig.1(a)).

Figure 1(b) shows the FEV₁ and slopes changes after salbutamol. FEV₁ increased significantly in the same way (p=0.583) in the three groups. The proportion of participants with an acute bronchodilator reversibility (defined as an FEV₁≥200 ml and ≥12% from the baseline value) (41) was 10.5, 20 and 4.5% in NO=, NO+, and NO- group, respectively, and this was similar in all three groups (p=0.317). However, Sₜₜ (Fig.1(c)) and Sₜₜ (Fig.1(d)) showed different behaviours in the groups: Sₜₜ and Sₜₜ did not change significantly in the NO= (p=0.063 and p=0.652, respectively) and the NO+ groups (p=0.284 and p=0.247, respectively) whereas, both slopes significantly decreased in the NO- group (p=0.007 and p<0.001, respectively).
Figure 1. Individual changes from baseline after bronchodilation for the total group NO-, NO=, and NO+ groups. Mean ± standard error of the mean and the statistical significances of changes are indicated for each group.

Discussion

In the present study, three different behaviours of $F_{E}NO$ response to $\beta_2$-agonist action were observed in patients with COPD and these responses most likely depend on the extent of the dilation process. A significant proportion of patients exhibited a $F_{E}NO$ decrease clearly associated with specific changes occurring in ventilation distribution indices that suggest a homogenous relief of airway obstruction down to lung periphery (i.e. down to intra-acinar airways). In contrast, a $F_{E}NO$ stability or increase was observed when bronchodilation appeared
to be more heterogeneous and to involve more proximal airways (i.e. up to terminal bronchioles).

FE\textsubscript{NO} is frequently used to monitor airway inflammation in asthma patients (2). However, beyond its usual function as a type 2 inflammatory marker, FE\textsubscript{NO} was also shown to be helpful in revealing different patterns of airway obstruction in asthma that were not apparent based on FEV\textsubscript{1}. In fact, we have recently documented several patterns of FE\textsubscript{NO} response to bronchodilation, likely dependent on the extent of the pre-dilation obstruction process (18). This appears to be true also for COPD patients. Despite no apparent effect of acute bronchodilation on FE\textsubscript{NO} levels in the entire COPD group, which is in line with previous data (27, 28), three different patterns of FE\textsubscript{NO} response to bronchodilation could be identified, considering the 11% threshold value previously reported as the short term variability of FE\textsubscript{NO} measured in COPD patients by chemiluminescence (39).

Specific changes in the ventilation distribution indices likely indicate that the extent and the degree of heterogeneity of the obstruction relief process, induced by β2-agonist action, are differing among the three groups. Indeed, the slope of phase III, assessing ventilation heterogeneities, may provide information about the site of airway alterations by the use of inert gases with different diffusivities (high for He and low for SF\textsubscript{6}). Theoretical basis of this feature (31) anticipated experimental results in the field of chronic rejection after lung transplant or COPD (42, 43) linking a decreased/increased slope with decreased/increased ventilation heterogeneities arising in specific locations: terminal bronchioles for He, intra-acinar airways for SF\textsubscript{6}.

In this study, a slope decrease after bronchodilation is associated with a homogeneous improvement of ventilation distribution induced by the bronchodilatory action of β2-agonist in a specific peripheral region. The homogeneous decrease of both He and SF\textsubscript{6} slopes with a trend to \(\Delta S_{SF6} > \Delta S_{He}\) observed in the NO- group supports the theory of an obstruction relief occurring
in the region extending up to the very peripheral airways (in the terminal bronchioles and acinar
airways (Fig.2(d)). This can amplify the NO back-diffusion process (25, 26), resulting in a
\( F_{\text{E}}\text{NO} \) value decrease.

The absence of a significant change of both slopes in the NO= and the NO+ groups suggests a
dilation process not detected by the SBWT. This could imply the involvement of more proximal
airways together with no change in \( F_{\text{E}}\text{NO} \) as observed in NO= group (Fig.2(a)) if the NO
production site is located mainly in peripheral airways in COPD as documented in asthma (44).
This could also suggest a more heterogeneous bronchodilation process likely due to the fact
that salbutamol may act more or less extensively on constricted small airways. This is illustrated
in Figures 2(b) and 2(c), which present two different dilation scenarios: extensive dilation of
neighbouring bronchioles lowering ventilation heterogeneities, thus decreasing slope
(Fig.2(c)), or dilation limited to the less constricted bronchioles, the other one remaining stable,
eventually increasing heterogeneities and slopes (Fig.2(b)). This was likely to occur as
suggested by the scattered distribution of slopes observed in this group. Again, if a large
proportion of NO is in fact produced in terminal conductive airways in COPD as it was
documented in asthma (44), it is worth noting that both scenarios should result in \( F_{\text{E}}\text{NO} \)
increase. Therefore, the coexistence of the two situations in pre-acinar small airways may result
in an average absence of slope change together with \( F_{\text{E}}\text{NO} \) increase as observed in the NO+ group.
Figure 2. Schematic representation of neighbouring bronchioles cross-sections before (solid line) and after dilation (dashed lines) in pre-acinar (left) and intra-acinar (right) airways. Bold lines represent airways with non-reversible obstruction. Panel (a): no reversibility, thus no change in $S_{He}$, $S_{SF6}$ and $F_{E}NO$. Panel (b): only the less constricted pre-acinar bronchiole is dilated, hence $S_{He}$ and $F_{E}NO$ increase. Panel (c): no intra-acinar reversibility ($S_{SF6}$ unchanged) and both pre-acinar bronchioles are dilated, hence $S_{He}$ decreases and $F_{E}NO$ increases; Panel (d): reversibility up to intra-acinar airways, both slopes decrease and $F_{E}NO$ decreases due to back-diffusion amplification.

To summarize, two different patterns of response to $\beta_2$-agonist action are identified in COPD patients related to the extent of the bronchodilation process: one pattern associated with a more heterogeneous proximal airway obstruction relief and another with a more homogeneous obstruction relief, down to the acinar airways. These two patterns can be easily detected in clinical practice by assessing $F_{E}NO$ evolution after acute bronchodilation. The reason behind the different depths of bronchodilation in response to the $\beta_2$-mimetic inhalation (i.e. different $\beta_2$-receptor densities along the airways, different aerosol distribution due to different patterns
of airway obstruction,...) remains to be elucidated. In any case, the decrease of both He and SF₆ slopes observed in the NO- group highly suggests that the bronchodilation induced by salbutamol can take place up to very peripheral airways in some COPD patients.

Such a peripheral action associated with a FₑNO decrease has already been reported in COPD patients but 3 hours after the inhalation of long acting β₂-agonists (LABA) though (30).

It is unlikely that the FₑNO changes observed after bronchodilation found in our study were influenced by the 10% FₑNO reduction induced by the repetition of forced manoeuvres during spirometry, sometimes observed in healthy subjects and asthma patients (24, 45, 46), but not always (23), and not in COPD patients so far. Moreover, in the only study using the current methodology to measure FₑNO in asthma patients, the FₑNO reduction was no longer significant 15 minutes after spirometry (24), which is approximately the moment when the post-bronchodilation FₑNO was measured in our study.

Regarding the mechanism involved in the FₑNO changes observed after salbutamol administration, some less likely action of the aerosol unrelated to change in peripheral airway obstruction should also be considered. An insult on the mucosa of the peripheral airways induced by the nebulization of salbutamol, resulting in a deterioration of the enzymatic machinery required for NO production or in mucus overproduction preventing NO diffusion into airway lumen in some patients, cannot be excluded but has never been documented as far as we are aware. Conversely, an activation of the NO enzymatic system is unlikely to explain the FₑNO increase observed in some patients after bronchodilation. In vitro studies have shown that several hours were required for NO to be released from cultured epithelial bronchial cells after initial stimulation with inflammatory cytokines (47).
Finally, the potential impact of the present data on clinical practice must be emphasized. It is generally accepted that FEV₁ is no longer an optimal parameter in COPD because it predominantly measures the degree of obstruction in large and intermediate airways while COPD is a disease affecting mainly the peripheral airways (48, 49). The present study shows, for the first time as far as we are aware, that this peripheral obstruction can be more or less extensively sensitive to the action of β₂-agonists. We suggest a simple procedure to identify patients with extensive β₂-agonist induced reversibility (up to the lung periphery) by measuring FENO values before and after bronchodilation. To-date, we have found no impact of the disease severity (GOLD grades, GOLD groups, Sp O₂) on the response patterns to β₂-agonist. However, their association with clinical outcomes such as symptoms, rate of exacerbation or the therapeutic approach should be prospectively investigated in larger cohorts of patients. It is tempting to speculate that patients belonging to the NO- group that displays a peripheral reversibility would be better candidates for therapies targeting also lung periphery such as LABA rather than LAMA likely to act more proximally (50).

In conclusion, three different FENO patterns were observed in response to β₂-agonists in COPD patients: a decrease reflecting a homogenous obstruction relief down to intra acinar airways and an increase or a stability likely associated with a more proximal heterogeneous dilation. As a result, a profile of airway obstruction with an extensive β₂-agonist response down to lung periphery is easily identified by FENO reduction after bronchodilation in 30 % of COPD patients. Future studies should focus on the short- and long-term variability overtime of these FENO patterns, their clinical relevance as well as on the impact of those bronchodilation patterns on gas exchanges.
Acknowledgements Section

Ethics approval and consent to participate:

The local ethics committee of the university hospital of Brussels approved the study on 12-02-2016 with the committee’s reference number B406201526529.

Availability of data and material:

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests:

SPB, AMa and AVM have nothing to disclose. Dr. Michils reports grants and personal fees from AstraZeneca, Chiesi and Novartis and personal fees from GlaxoSmithKline, outside the submitted work.

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Author’s contributions:

Conception, design and protocol development: SPB AMi AVM. Data collection: SPB AVM. Analysis and interpretation of the data: SPB AMi AMa AVM. Writing of the manuscript: SPB AMi AMa AVM. Critical review of the manuscript: SPB AMi AMa AVM. All authors approved the manuscript to be published.

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References


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