Effect of exhalation flow rates and level of nitric oxide output on accuracy of linear approximation of pulmonary nitric oxide dynamics

Tuomas Karvonen and Lauri Lehtimäki

This is the Accepted Manuscript version of an article accepted for publication in The Journal of Breath Research. IOP Publishing Ltd is not responsible for any errors or omissions in this version of the manuscript or any version derived from it. The Version of Record is available online at https://doi.org/10.1088/1752-7163/abf3ab

Effect of exhalation flow rates and level of nitric oxide output on accuracy of linear approximation of pulmonary nitric oxide dynamics

Tuomas Karvonen[1] and Lauri Lehtimäki[1,2]

[1] Tampere University Respiratory Research Group, Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland

[2] Allergy Centre, Tampere University Hospital, Tampere, Finland

Email: lauri.lehtimaki@tuni.fi

Keywords: Two-compartment model, breath tests, nitric oxide

Abstract

The method of Tsoukias and George (T&G) is a commonly used linear approximation of pulmonary nitric oxide (NO) dynamics that can be used to calculate bronchial NO output ($J_{aw}NO$) and alveolar NO concentration (C_ANO). We aimed to investigate how flow rate range in exhaled NO measurements and levels of pulmonary NO parameters affect the accuracy of the T&G method.

This study has three parts. 1) A theoretical part demonstrating how different exhalation flow rates and NO parameter levels affect the accuracy of the T&G method, 2) testing how exhalation flow rate range affects the method in a sample of asthmatic and healthy subjects, and 3) a meta-analysis of published literature to test whether minimum flow rate has an association with the NO parameter values.

We found that both the chosen exhalation flow rates and magnitude of the pulmonary NO parameters affect the accuracy of the T&G method. Underestimation of $J_{aw}NO$ increased with lower flow rates and higher

bronchial diffusion factor of NO ($D_{aw}NO$), while overestimation of C_ANO increased with higher $D_{aw}NO$ and bronchial wall NO concentration ($C_{aw}NO$) and lower C_ANO. Of the NO parameters, C_ANO was the most prone to bias and high $D_{aw}NO$ was the most significant factor causing the bias. Furthermore, we found that using 40 ml s^{-1} as the lowest flow rate in our sample and 50 ml s^{-1} in the meta-analysis compared to 100 ml s^{-1} resulted in higher C_ANO, but $J_{aw}NO$ was not statistically significantly affected.

We have provided objective evidence that not only the flow rates used but also the magnitude of NO output in the test subjects affect the accuracy of the T&G method. We suggest that flow rates below 100 ml s^{-1} should not be used with the T&G method to maintain accuracy.

Introduction

Fractional exhaled nitric oxide (F_ENO) is a marker of eosinophilic inflammation in the lungs (Berry et al 2005). F_ENO-measurement at one exhalation flow rate of 50 ml s^{-1} is standardized and used in diagnostics and clinical management of asthma (GINA Report 2018, National Institute for Health and Clinical Excellence 2017). However, the standard F_ENO measurement reflects NO dynamics in the central conducting airways and is relatively insensitive to changes in peripheral airways (Lehtimäki et al 2020). Performing F_ENO-measurement at multiple flow rates (extended F_ENO-measurement) allows calculation of flow independent NO parameters according to the two-compartment model of pulmonary NO exchange dynamics (Tsoukias and George 1998). The NO parameters have several promising applications in many pulmonary diseases. A major advantage compared to the single flow rate F_ENO measurement is the capability to separate bronchial and alveolar sources of NO. For instance, NO parameters provide promising markers for parenchymal lung inflammation in interstitial lung diseases and markers for tissue damage in both alveolar and bronchial regions (Lehtimäki et al 2020). In addition, the NO parameters can be used to detect small airway inflammation in asthma, which remains undetected by the conventional F_ENO measurement.

In the two-compartment model, the lungs are divided into two different regions: an expansible alveolar region describing the compartment involved in respiratory gas exchange and a rigid bronchial region that participates in respiratory gas conduction. The exponential equation of the two-compartment model is capable of predicting F_ENO value at a given flow rate (Equation 1):

$$F_ENO = C_{aw}NO + (C_ANO - C_{aw}NO)e^{-\frac{D_{aw}NO}{Ve}} \qquad \textbf{Equation 1.}$$

where $C_{aw}NO$ is bronchial mucosal NO concentration (ppb), C_ANO is NO concentration in alveolar air (ppb), $D_{aw}NO$ is bronchial wall diffusing capacity of NO (pl s^{-1} ppb^{-1}), and Ve is exhalation flow rate (ml s^{-1}).

F_ENO consists of two components. First, alveolar air containing low concentration of NO (C_ANO) leaves the alveolar region during exhalation and

gets then enriched by NO in the bronchial region. NO concentration is usually markedly higher in the bronchial wall mucosa compared to air in the lumen, and the direction of NO flux is therefore in practice always from bronchial wall to lumen.

The enrichment of alveolar air with bronchial NO during exhalation depends on the flow-independent NO parameters of the bronchial region ($D_{aw}NO$ and $C_{aw}NO$) and C_ANO itself. The flow rate dependence of F_ENO derives from the time that the air from the alveolar compartment spends in the bronchial region to collect more NO from the bronchial wall mucosa. This can also be understood mathematically when looking at the two-compartment model's exponential equation (Equation 1): as flow rate approaches infinity, F_ENO approaches C_ANO ($\lim_{Ve\to\infty} F_ENO = C_ANO$), and when flow rate approaches zero, F_ENO approaches $C_{aw}NO$ ($\lim_{Ve\to 0} F_ENO = C_{aw}NO$).

Several mathematical methods have been introduced for the estimation of the NO parameters. Some of the methods utilize the nonlinear equation of the 2CM and are able to estimate all NO parameters (Högman et al 2002, Eckel et al 2014, Silkoff et al 2000), while others utilize linear approximations of the nonlinear equation. The advantage of linear methods is that they are simpler to implement mathematically but with a price of being able to solve only two parameters (Pietropaoli et al 1999, Tsoukias et al 2001). In the literature, linear methods are the distinctly most frequently used techniques in NO parameter estimation (Molshatski and Eckel 2017). Of the linear methods, the most frequently used is the method introduced by Tsoukias and George (the T&G method).

Theory behind the method of Tsoukias & George (T&G)

In the T&G method, a linear first-order approximation (Equation 2) of the exponential part of equation 1 is used: (George et al 2004)

$$F_ENO = C_{aw}NO + (C_ANO - C_{aw}NO)e^{-\frac{D_{aw}NO}{Ve}} \qquad \textbf{Equation 1.}$$

$$e^{-\frac{D_{aw}NO}{Ve}} \approx 1 - D_{aw}NO/Ve \qquad \textbf{Equation 2.}$$

yielding the first-order approximation of equation 1**:**

$F_ENO = C_ANO + (C_{aw}NO - C_ANO) * D_{aw}NO/Ve$ **Equation 3.**

When equation 3 is multiplied by flow rate on both sides, the equation describes NO output in the exhaled breath at the given flow rate:

$V_{NO} = C_ANO * Ve + (C_{aw}NO – C_ANO) * D_{aw}NO$ **Equation 4.**

As $J_{aw}NO = (C_{aw}NO - C_ANO) * D_{aw}NO$, equation 4 is further reduced to (Horvath et al 2017):

$V_{NO} = C_ANO * Ve + J_{aw}NO$ **Equation 5.**

where V_{NO} is NO output ($F_ENO * Ve$) and $J_{aw}NO$ is bronchial NO flux (pl s^{-1}).

When a linear regression line is set between NO output (V_{NO}, pl s^{-1}) and exhalation flow rate (Ve, ml s^{-1}), the intercept of the regression line yields an estimate of $J_{aw}NO$ and the slope yields an estimate of C_ANO (Equation 5).

The first-order approximation (Equation 2) is based on a mathematical fact that $e^x \approx 1-x$ when x is small. For the first-order approximation to hold, the quantity of $-D_{aw}NO/Ve$ must therefore be small. This assumption is satisfied when the flow rate is high and $D_{aw}NO$ is relatively small. Using too low flow rates may thus violate this assumption and result in falsely low and high estimates of $J_{aw}NO$ and C_ANO, respectively (Figure 1). When considering subjects with higher $D_{aw}NO$, the flow rate should be higher to achieve similar quantity of $-D_{aw}NO/Ve$ as with subjects with lower $D_{aw}NO$. Thereby, when same flow rate range is used, subjects with higher $D_{aw}NO$ (e.g subjects with

asthma) will always have more biased results (underestimation of $J_{aw}NO$ and overestimation of $C_A NO$) compared to the subjects with lower $D_{aw}NO$ (e.g. healthy subjects). This may result in false differences in $C_A NO$ between populations with different DawNO. George *et al*. instructed in their review that the approximation holds when Ve is large compared to $D_{aw}NO$ ($Ve \approx 5*D_{aw}NO$) which is usually achieved at flow rates around 50 ml s^{-1} (George et al 2004). This is the lowest used flow rate in most studies using linear methods in NO parameter estimation (Molshatski and Eckel 2017). However, this instruction was meant for healthy subjects, whose $D_{aw}NO$ is low compared to asthmatics for example. The current ERS/ATS recommendation suggests the usage of at least 100 ml s^{-1} as the lowest flow rate for the linear methods (Horvath et al 2017). However, this recommendation is based on expert opinion and there is no previous thorough modeling on how the selected flow rates affect the accuracy of the T&G method in practice, and whether the range of suitable flow rates is different in different kinds of pulmonary diseases. This information is needed to improve the standardization of the use of the T&G method.

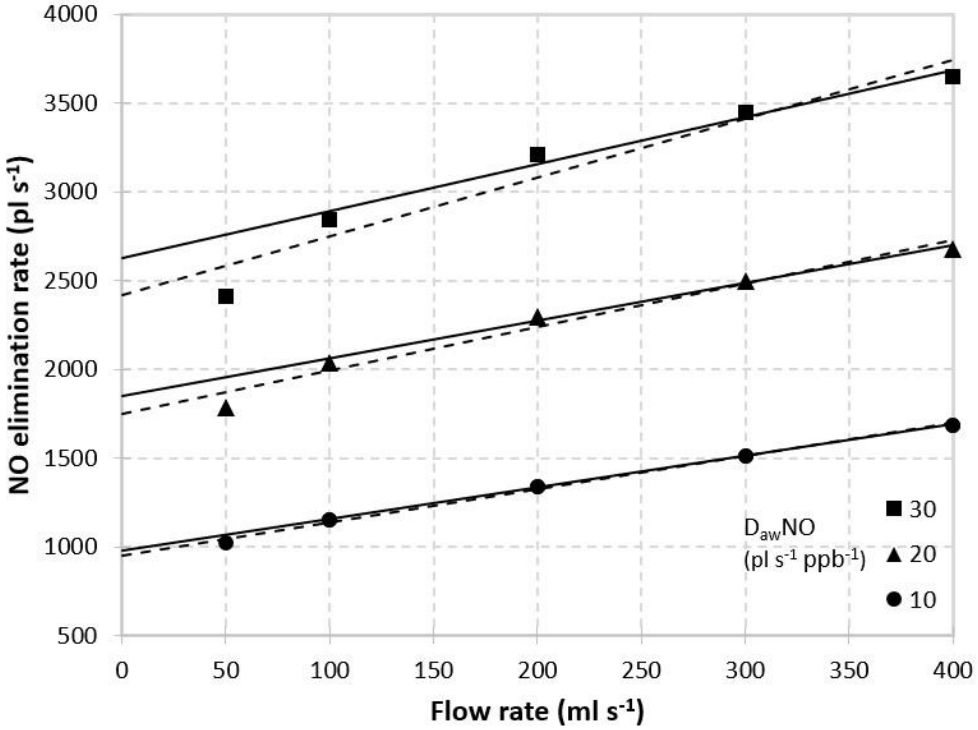

Figure 1. NO output plotted against exhalation flow rate in examples with various $D_{aw}NO$ values (10, 20 and 30 pl s^{-1} ppb^{-1}). Other parameters are set as in typical healthy subject with C_ANO of 1.66 ppb and CawNO of 105 ppb (Högman et al 2017). The solid regression line is set between NO output and flow rate using flow rates 100 - 400 ml s^{-1}, whereas the dotted regression line is set using flow rates 50 - 400 ml s^{-1} to visualize how the slope and intercept (estimates of C_ANO and $J_{aw}NO$, respectively) are affected by the applied flow rate range with different $D_{aw}NO$ values. As linearity of the relation begins to break at approximately 100 ml s^{-1}, setting regression line including lower flow rates results in falsely low intercept ($J_{aw}NO$) and high slope (C_ANO) compared to a regression line based on the more linear part of the relation. Higher $D_{aw}NO$ results in less linear plot and thus more error in the parameter estimation.

Aim of the study

This study aimed to investigate how the chosen flow rate range and levels of pulmonary NO parameters affect the accuracy of the T&G method and to provide objective evidence for methodological recommendations on the

T&G method. This study consists of three parts. 1) In the first part, we will demonstrate how different flow rates and NO parameter levels influence the accuracy of the T&G method in NO parameter estimation. 2) In the second part, we will test whether real life observations support the theory. We will test whether using a lower flow rate range yields different NO parameter estimates in healthy and asthmatic subjects. 3) In the third part, we will conduct a meta-analysis of published literature to investigate whether including 50 ml s^{-1} as the lowest flow rate in the T&G method has a noticeable effect compared to 100 ml s^{-1} as the lowest flow rate.

Methods

Demonstration on the effects of NO parameters and selected flow rates on NO parameter estimation

NO parameter estimates obtained using the T&G method were compared to the true values of the NO parameters obtained using the 2CM's nonlinear equation. First, the 2CM's nonlinear equation (Equation 1) was used to calculate F_ENO at flow rates of 10, 30, 50, 100, 200, 300 and 400 ml s^{-1} based on C_ANO, $C_{aw}NO$ and $D_{aw}NO$ according to the reference values by Högman *et al* (Högman et al 2017). Then one NO parameter at a time was changed within a wide range ($D_{aw}NO$ from 1 to 45 pl s^{-1} ppb^{-1}, $C_{aw}NO$ from 1 to 500 ppb and C_ANO from 1 to 15 ppb) while the other two parameters were kept constant. The corresponding NO outputs (V_{NO}) were then calculated, and the T&G method was applied on multiple flow rate ranges (30 – 400, 50 – 400, 100 – 400 and 200 – 400 ml s^{-1}) to estimate $J_{aw}NO$ and C_ANO. The T&G estimates of $J_{aw}NO$ and C_ANO were then compared to the true $J_{aw}NO$ (= [$C_{aw}NO$ - C_ANO] * $D_{aw}NO$) and true C_ANO by calculating a proportion between the T&G estimate and true value and plotting this proportion against each of the NO parameters.

Different flow rate ranges in healthy and asthmatic subjects

The T&G method was applied to F_ENO-measurements in healthy non-smoking adults and steroid-naïve adult asthmatics from a previously published study (Lehtimäki et al 2001). F_ENO was available at flow rates of 40, 100, 170 and 370, ml s^{-1}, and the T&G was applied by using two different flow rate ranges: 40 – 370 ml s^{-1} and 100 – 370 ml s^{-1}. A correlation coefficient

of < 0.9 or negative C_ANO were exclusion criteria to assess the quality of the F_ENO-measurements (V_{NO} vs Ve -plot). Box plots were drawn for $J_{aw}NO$ and C_ANO with different flow rate ranges and nonparametric Wilcoxon's test or paired T-test were used to compare distributions, depending on the normality of the distributions (Shapiro-Wilk's test).

Meta-analysis on previously published results

A meta-analysis was conducted to assess whether the use of 50 ml s^{-1} as the lowest flow rate results in a statistically significant difference compared to 100 ml s^{-1} as the lowest flow rate in the T&G method. A previously published systematic review and meta-analysis including all studies that reported NO parameters for both asthmatic and healthy subjects was used as the data source (Karvonen and Lehtimäki 2019). Steroid naïve-asthmatics were chosen to represent asthmatic subjects as this group was less heterogeneous than the steroid-treated group.

To conduct the meta-analysis, the lowest flow rate was set as a dichotomous categorical moderator (categories: 50 ml s^{-1} and 100 ml s^{-1}). A subgroup meta-analysis was then conducted to estimate the effect size for each level of the moderator and to test for statistically significant inter-group differences. A mixed model, with DerSimonian-Laird approach as an inter-study variation estimator, was used to calculate the differences between the two categories of the moderator (Anonymous 2019). A pooled estimation of the inter-study variance across the categories of the moderator was chosen (i.e. same amount of inter-study variance, same τ^2-value). A pooled inter-study variance estimation is recommended over separate estimation within each category of a moderator when the number of studies is balanced, and the residual inter-study variances are not heteroscedastic across categories of the moderator (María Rubio-Aparicio et al 2020).

The difference between using these two flow rates as the lowest was illustrated by drawing forest plots. The effect size for each subgroup was calculated separately with mixed model but τ^2-value was set according to the pooled estimation of inter-study variance performed earlier in the meta-analysis. Meta-analysis was conducted, and forest plots were drawn using R version 3.4.3 (R Core Team 2016) and the Metafor package (Viechtbauer 2010).

Results

Demonstration on the effects of NO parameters and selected flow rates on NO parameter estimation

$J_{aw}NO$

When $J_{aw}NO$ was estimated using the T&G method and compared to true $J_{aw}NO$, it was found that results strongly depended on $D_{aw}NO$ (Figure 2). Another factor that affected the results was the used range of flow rates: a lower flow rate range resulted in more deviation from true $J_{aw}NO$. Taken together, the magnitude of underestimation when using the T&G method to calculate $J_{aw}NO$ increases with higher absolute $D_{aw}NO$ and lower flow rates used. Other NO parameters (C_ANO and $C_{aw}NO$) had no effect on the proportion between the T&G estimate of $J_{aw}NO$ and true $J_{aw}NO$.

C_ANO

When C_ANO was estimated using the T&G method, all NO parameters affected the proportion of the T&G C_ANO from true C_ANO (Figure 3). Higher $D_{aw}NO$ and $C_{aw}NO$ caused more overestimation of C_ANO. On the other hand, higher C_ANO resulted in less biased results. Of the parameters, $D_{aw}NO$ seemed to be the clearly most significant factor causing bias. The higher was the lowest flow rate used the smaller was the overestimation of C_ANO when using the T&G.

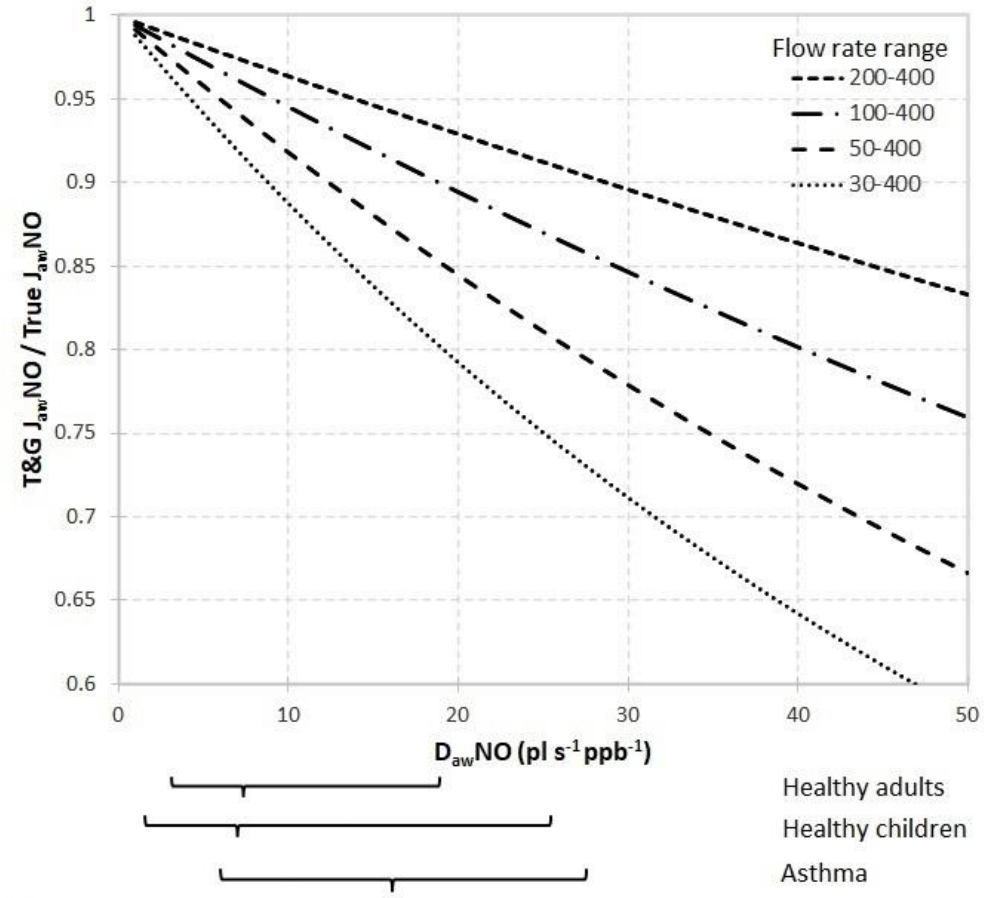

Figure *2*. The relation between the T&G estimate of $J_{aw}NO$ and true $J_{aw}NO$ plotted against $D_{aw}NO$ using different flow rate ranges. Higher values of $D_{aw}NO$ and lower flow rate ranges resulted in higher error in estimates of $J_{aw}NO$. Example distributions (mean and range from 5th to 95th percentile) of $D_{aw}NO$ of healthy adults (Högman et al 2017), healthy children (Högman et al 2017) and patients with asthma (Karvonen and Lehtimäki 2019) are indicated with brackets below the x-axis to help the reader to understand the magnitude of error in different populations. For example, in a typical patient with asthma ($D_{aw}NO$ 16 pl s^{-1} ppb^{-1}) the flow rate range of 30 – 400 underestimates $J_{aw}NO$ roughly 13 %, while in a typical healthy adult ($D_{aw}NO$ 8 pl s^{-1} ppb^{-1}) the flow rate range of 200 – 400 ml s^{-1} underestimates $J_{aw}NO$ only by 3 %.

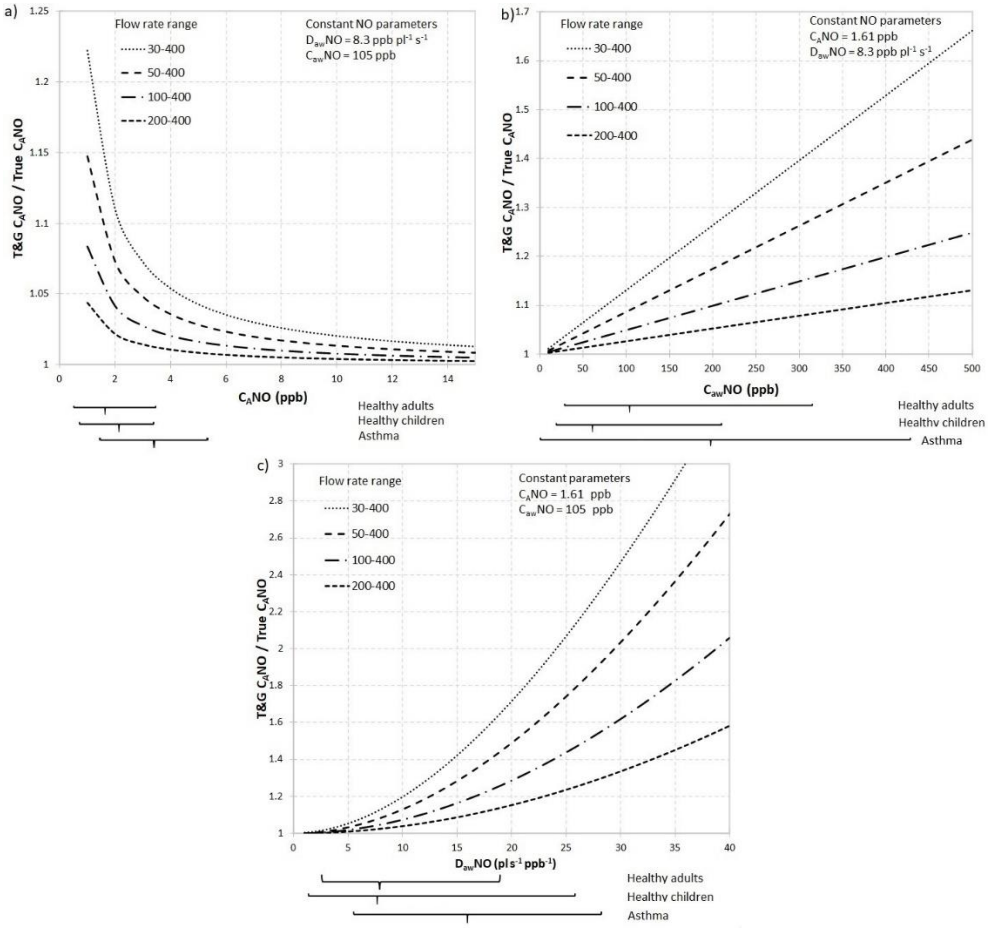

Figure 3. The relation of the T&G estimate of C_ANO and true C_ANO plotted against true C_ANO (a), $C_{aw}NO$ (b) and $D_{aw}NO$ (c). Higher values of $D_{aw}NO$ and $C_{aw}NO$ and lower flow rate ranges resulted in more error. Higher C_ANO resulted in less biased results compared to true C_ANO. $D_{aw}NO$ seemed to have the greatest effect on the estimation error. Distribution (mean and range from 5th to 95th percentile) of the NO parameters for healthy adults (Högman et al 2017) and children (Högman et al 2017) and asthmatics (Karvonen and Lehtimäki 2019) are marked with brackets below the x-axis to give a perspective of the magnitude of error in different study populations. For example, in a typical patient with asthma ($D_{aw}NO$ 16 pl s^{-1} ppb^{-1}) the flow rate range of 30 – 400 overestimates C_ANO by 47 %, while in a typical healthy adult ($D_{aw}NO$ 8 pl s^{-1} ppb^{-1}) the flow rate range of 200 – 400 ml s^{-1} overestimates C_ANO by only about 3%.

Different flow rate ranges in healthy and asthmatic subjects

Overall, 22 healthy and 22 asthmatic subjects were included in the analyses (Lehtimäki et al 2001). Using flow rate range of 40 – 370 ml s^{-1} compared to 100 – 370 ml s^{-1} produced no statistically significant difference in $J_{aw}NO$ or C_ANO in healthy subjects ($J_{aw}NO$: 455 (403/720) vs 446 (388/752) pl s^{-1}, p = 0.291 and C_ANO: 1.4 (0.9/1.7) vs 1.4 (1.0/1.7) ppb, p = 0.355) (median (1^{st}/3^{rd} quartile)) (Figure 4A and 4B). However, the use of the lower flow rate range yielded lower $J_{aw}NO$ (1124 (502/1832) vs 1188 (609/1896) pl s^{-1}, p = 0.006) and higher C_ANO (1.7 (1.4/2.7) vs 1.5 (1.2/2.3) ppb, p = 0.006) in the asthmatic subjects (Figure 4C and 4D).

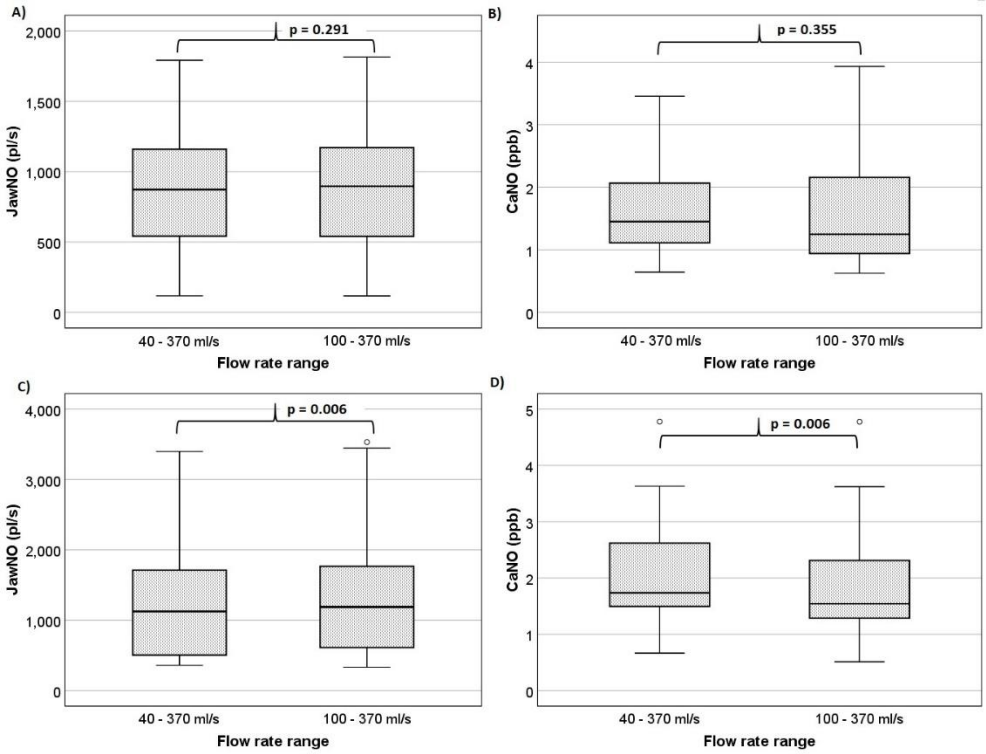

Figure 4. NO parameters in asthmatic and healthy subjects estimated with the T&G method using two different flow rate ranges: A) $J_{aw}NO$ and B) C_ANO of healthy adults, C) $J_{aw}NO$ and D) C_ANO of asthmatic subjects.

Meta-analysis on previously published results

9 studies were included in the meta-analysis. Of the 9 studies, 5 used 50 ml s^{-1} and 4 used 100 ml s^{-1} as the lowest flow rate.

Studies using 50 ml s^{-1} as the lowest flow rate reported $J_{aw}NO$ estimates with no significant difference to studies with 100 ml s^{-1} as the lowest flow rate in healthy subjects (difference 50 – 100 ml s^{-1}: -125 [-310 – 58] pl s^{-1}, p = 0.18) (average difference [CI 95% lower bound – upper bound]) or in asthmatic subjects (-345 [-1147 – 457] pl s^{-1}, p = 0.40) (Figures 5 and 6).

C_ANO in healthy subjects seemed to be higher in studies with 50 ml s^{-1} as the lowest flow rate (1.57 [-0.05 – 3.2] ppb, p = 0.06), but the difference was not quite statistically significant. In asthmatic subjects, C_ANO was higher in

studies using 50 ml s^{-1} as the lowest flow rate (4.0 [1.12 – 6.89] ppb, $p < 0.01$) (Figures 5 and 6).

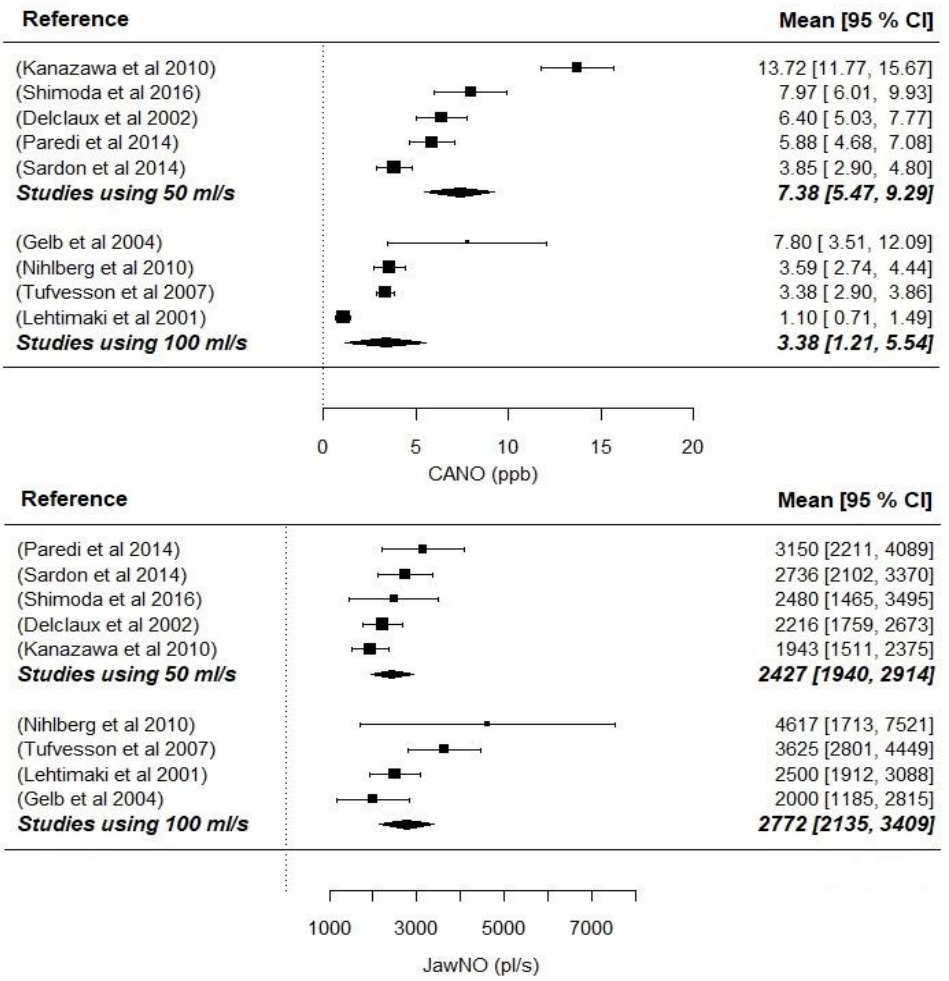

Figure 5. Forest plots showing distribution of the NO parameters in asthmatic subjects in studies using 50 ml s^{-1} or 100 ml s^{-1} as the lowest flow rate. There was no statistically significant difference in $J_{aw}NO$ between the groups (-345 [-1147 – 457] pl s^{-1}, $p = 0.40$) but C_ANO was higher in studies with 50 ml s^{-1} as the lowest flow rate (4.0 [1.12 – 6.89] ppb, $p < 0.01$).

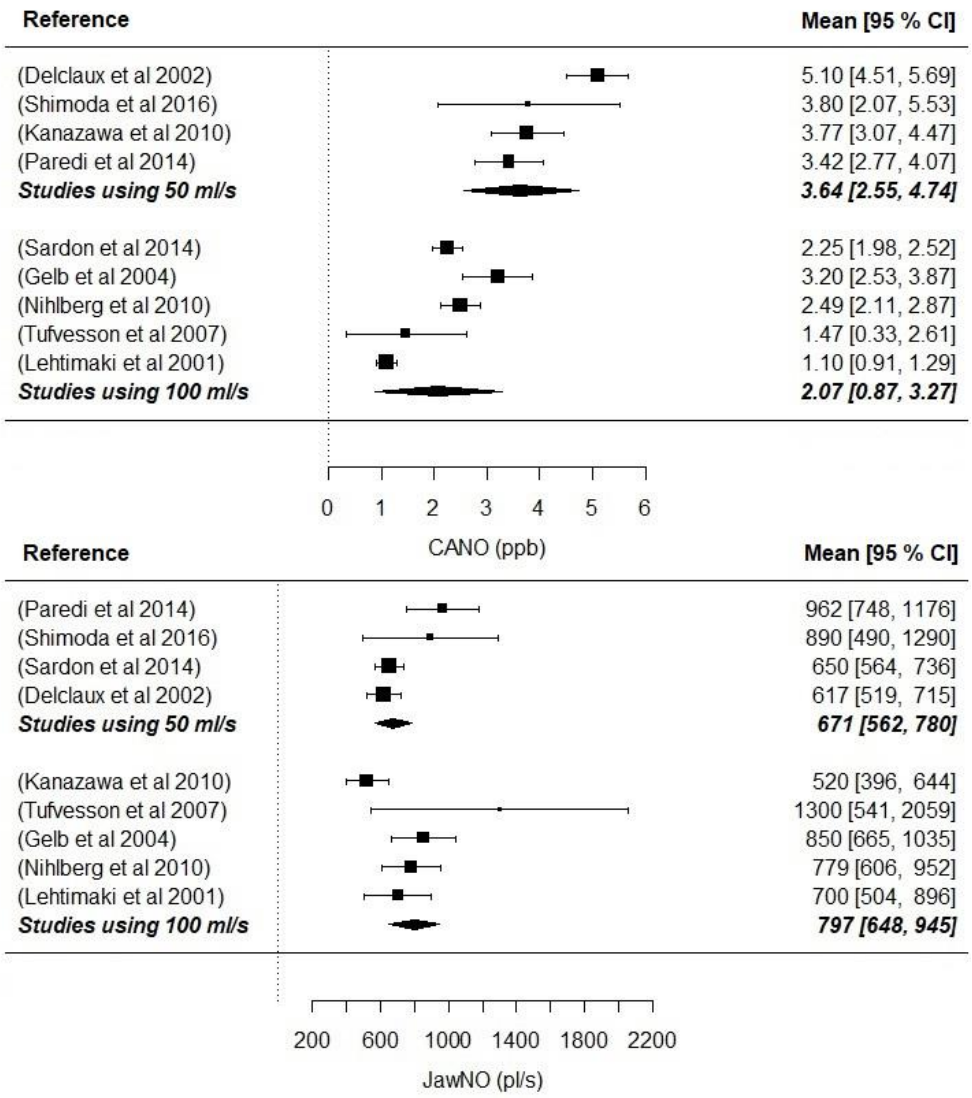

Figure *6*. Forest plots showing distribution of the NO parameters in healthy subjects in studies using 50 or 100 ml s^{-1} as the lowest flow rate. No statistically significant differences were found between the groups but $J_{aw}NO$ seemed to be lower (-125 [-310 – 58] pl s^{-1}, p = 0.18) and C_ANO higher (1.57 [-0.05 – 3.2] ppb, p = 0.06) in studies using 50 ml s^{-1} as the lowest flow rate.

Discussion

We demonstrated that the accuracy of the T&G method is not only dependent on the flow rate range used but also on the magnitude of the flow independent NO parameters. When using the T&G method, underestimation on $J_{aw}NO$ increases not only with lower flow rates, but also with higher values of $D_{aw}NO$. Simultaneously, overestimation of C_ANO increases with lower flow rates in addition to higher values of $C_{aw}NO$ and $D_{aw}NO$, while this error slightly reduces with higher values of C_ANO. Our real-life observations showed that in patients with asthma (higher $C_{aw}NO$ and $D_{aw}NO$), inclusion of a low flow rate of 40 ml s^{-1} makes a significant difference to the results of the T&G method when compared to 100 ml s^{-1} as the lowest flow rate. However, this difference was not found in healthy subjects (lower $D_{aw}NO$ and $C_{aw}NO$). Finally, this same phenomenon was found in a meta-analysis showing that in subjects with asthma, inclusion of 50 ml s^{-1} as the lowest flow rate for the T&G method overestimates C_ANO compared to studies with 100 ml s^{-1} as the lowest flow rate.

Agreement with the literature

This is the first thorough study on the aspects affecting the accuracy of the T&G method. There are no previous studies systematically assessing the effect of the magnitude on NO parameters on the accuracy of the T&G method, but there are some studies reporting similar results to our study regarding the effect of flow rates. Pedroletti *et al.* found loss of linearity in the NO output vs flow rate curve at flow rates below 50 ml s^{-1} in healthy subjects but already below 99 ml s^{-1} in asthmatic subjects (Pedroletti et al 2002). Robroecks *et al.* found that using 30 ml s^{-1} as the lowest flow rate instead of 50 ml s^{-1} results in higher C_ANO in children with asthma (Robroeks et al 2010). Also, Chladkova *et al.* found that C_ANO was up to 67 % higher in subjects with asthma and 28 % higher in subjects with allergic rhinitis when flow rate ranges 50 – 250 ml s^{-1} was compared to the range of 150 - 250 ml s^{-1} (Chladkova et al 2012). Simultaneously, $J_{aw}NO$ was increased by 50 % and 17 %, respectively. The authors concluded that linearity was preserved better when 150 ml s^{-1} was used as the lowest flow rate.

However, not all studies noticed a significant difference when 50 ml s^{-1} was used as the lowest flow rate. Heijkenskjöld *et al.* compared two flow rate ranges (50–200 ml s^{-1} vs 100–300 ml s^{-1}) in young asthmatic subjects (10 – 19 years) and found no difference in C_ANO obtained using these flow rate ranges (Heijkenskjöld-Rentzhog et al 2012).

The current literature has results supporting our findings that 100 ml s^{-1} should be used as the lowest flow rate in the T&G method and that uncertainty is greater in C_ANO than in $J_{aw}NO$, but some inconsistency was also found. The inconsistency is possibly due to different levels of the NO parameters, especially $D_{aw}NO$, in different study populations. Our findings on how the magnitude of NO parameters themselves affect the accuracy of the T&G method give mechanistic explanation on why previous studies have reported different breaking points for the linearity of V_{NO} vs Ve curve in different subject categories.

Axial back diffusion of NO affecting accuracy of the T&G method

The two-compartment model assumes the airways as an even, rigid, cylinder-shaped tube. However, in real airways, the total cross-sectional surface area increases with every new generation of bronchi. As the total surface area increases, the velocity of air flow decreases simultaneously. It is hypothesized that the decrease in the air flow velocity is sufficient enough to allow axial back-diffusion of NO from the more NO-rich bronchial compartment into the alveolar compartment. The two-compartment model is thus believed to overestimate $J_{aw}NO$ and underestimate C_ANO because of neglecting the axial back-diffusion of NO.

Condorelli *et al*. introduced the trumpet model of airways with axial diffusion (TMAD) (Condorelli et al 2007) to compensate for the effects of axial back diffusion in the conventional two-compartment model by using correction factors. However, the correction factors of this model only apply to subjects without airway obstruction and should not be used in subjects with e.g. COPD or asthma.

Lower flow rates seemed to cause more biased results in asthmatic subjects than in healthy subjects. One explanation could be that in healthy subjects with no obstruction, axial back-diffusion of NO is more significant and masks

the bias caused by low flow rate used in the T&G method (i.e. low flow rate causes lower estimate of $J_{aw}NO$, whereas neglecting back-diffusion of NO causes higher estimate of $J_{aw}NO$). However, in asthmatic subjects with obstruction, back-diffusion of NO is believed to be less significant and hence bias caused by low flow rates is emphasized. All in all, a more plausible explanation is that asthmatics have higher $D_{aw}NO$ that is strongly associated with higher bias in NO parameter estimation with the T&G method.

Strengths and limitations

We have investigated the effect of flow rates and NO parameters in the T&G method by three different approaches (i.e. mathematical demonstration, results from our previous study and meta-analysis) that all provided concordant results. We therefore think that we have quite robust evidence that can be used in future recommendations on the use of the T&G method. However, there are also some limitations to the study. The two-compartment model is a mathematical model describing the pulmonary NO exchange dynamics, but the real lung and relation between NO parameters and the T&G method may be much more complex. In addition, more error is added to the model when F_ENO-measurements are performed in real subjects with possible difficulties in performing the measurement. For instance, some subjects may find a higher flow rate range more challenging to perform and this may be emphasized in small children and cause bias at high flow rate ranges. Another limitation of this study is the relatively small sample size of healthy and asthmatic subjects. There are also some limitations regarding the meta-analysis. It is not intended as a systematic review of the current literature but rather act as a sample of the current literature. Interstudy variation is considerable and we identified multiple known and potential confounding factors. Methodological aspects included different flow rates that are known to produce different results. There are also other methodological aspects that may cause bias as the extended F_ENO measurement lacks technical standardization (e.g. different analyzers, calibration protocols, time of measurement, ambient air NO). There are also no official guidelines for F_ENO measurement at other flow rates than 50 ml s^{-1}. The guidelines for 50 ml s^{-1} can be quite well applied to most other flow rates. However, extremely low and high flow rates (e.g. 10 and 400 ml s^{-1}) might benefit from their own guidelines as the definition of NO

concentration plateau at 50 ml s^{-1} might be difficult to apply to these extreme flow rates (e.g. minimum plateau duration of 3 seconds). However, their significance in the extended F_ENO measurement is unknown. Also, different groups of asthmatic subjects are highly heterogeneous, which likely contributed to the interstudy variation.

Also, we only observed the lowest flow rate used while possible differences in the other used flow rates were ignored in the analysis. The number of included studies was also quite small. However, of all the diseased study populations, the NO parameters are calculated most frequently in asthmatic subjects, making them currently the best option for such a meta-analysis.

Recommendation for future studies

We have provided objective evidence to support the current ERS/ATS expert opinion based recommendation (Horvath et al 2017) that no lower flow rates than 100 ml s^{-1} should be used in NO parameter estimation with the T&G method. In practice, there is always some underestimation of $J_{aw}NO$ and overestimation of C_ANO, but the magnitude of error is determined by two factors: used flow rate range and magnitude of NO parameters.

$D_{aw}NO$ was the most significant NO parameter affecting the accuracy of the T&G method in NO parameter estimation and the degree of bias caused by high $D_{aw}NO$ can be only reduced by using a high enough flow rate range. Theoretically, 50 ml s^{-1} should be sufficient for healthy subjects (low $D_{aw}NO$) based on our findings in healthy subjects and meta-analysis. However, as in clinical practice we cannot know in advance whether the subjects are healthy or not, 100 ml s^{-1} should always be used as the lowest flow rate in the T&G method. A coherent recommendation to all subject groups also makes the T&G method more standardized and results between different study groups more comparable. The main advantage of the T&G method is its ability to calculate C_ANO, as $J_{aw}NO$ is highly correlated with the well-standardized F_ENO_{50} and thus probably does not provide additional clinical value. Of the two parameters, C_ANO is much more prone to bias when estimated using the T&G method, advocating the use of high enough flow rate ranges. In the literature, the T&G method has shown to yield higher C_ANO and lower $J_{aw}NO$ estimates in healthy subjects when compared to non-linear methods when 100 ml s^{-1} was used as the lowest flow rate (Karvonen et al 2017). The

difference to non-linear methods could be explained with bias caused by linear approximation, even if the lowest flow rate was 100 ml s^{-1} and $D_{aw}NO$ was relatively low in the healthy subjects (median 7.4 pl s^{-1} ppb^{-1}). This may suggest that the T&G method is subtle to some bias even in ideal conditions. Another serious limitation of the T&G is that the NO parameter estimates are always more biased with populations with higher $D_{aw}NO$. This may result in false differences especially in C_ANO when populations with different $D_{aw}NO$ are compared. Therefore, we encourage the use of nonlinear methods as an alternative to the T&G method. The nonlinear methods lack the bias caused by linear approximation.

Conclusions

We have provided objective and robust evidence that not only the flow rates used but also the magnitude of NO output in the test subjects affect the accuracy of the T&G method. Underestimation on $J_{aw}NO$ increases with too low flow rates and with higher values of $D_{aw}NO$. The corresponding overestimation of C_ANO increases with too low flow rates and with higher values of $D_{aw}NO$ and $C_{aw}NO$ but decreases with higher values of C_ANO. As in clinical practice, we do not know in advance the NO output of the test subject, based on our data flow rates below 100 ml s^{-1} should not be used with the T&G method.

Ethical statement

In this study, we used data from subjects from a previous study. The previous study was approved by the ethics committee of Tampere University Hospital (98140) and all subjects gave their written informed consent.

Funding

The study was supported by Foundation of the Finnish Anti-Tuberculosis Association (https://tb-foundation.org/), Tampere Tuberculosis Foundation (http://www.tuberkuloosisaatio.fi/), The Research Foundation of the Pulmonary Diseases (https://www.hengitysliitto.fi/en/), Tampere University Hospital Support Foundation (https://www.taystuki.fi), and Competitive State Research Financing of the Expert Responsibility area of Tampere University Hospital.

Conflicts of interest

We declare no conflicts of interest.

References

Berry M A, Shaw D E, Green R H, Brightling C E, Wardlaw A J and Pavord I D 2005 The use of exhaled nitric oxide concentration to identify eosinophilic airway inflammation: an observational study in adults with asthma Clinical & Experimental Allergy **35** 1175-1179

Chladkova J, Senkerik M, Havlinova Z, Krcmova I and Chladek J 2012 Alveolar concentration and bronchial flux of nitric oxide: two linear modeling methods evaluated in children and adolescents with allergic rhinitis and atopic asthma. Pediatr.Pulmonol. **47** 1070-1079

Condorelli P, Shin H, Aledia A S, Silkoff P E and George S C 2007 A simple technique to characterize proximal and peripheral nitric oxide exchange using constant flow exhalations and an axial diffusion model. J.Appl.Physiol. **102** 417-425

Delclaux C, Mahut B, Zerah-Lancner F, Delacourt C, Laoud S, Cherqui D, Duvoux C, Mallat A and Harf A 2002 Increased nitric oxide output from alveolar origin during liver cirrhosis versus bronchial source during asthma. American Journal of Respiratory & Critical Care Medicine **165** 332-337

Eckel S P, Linn W S, Berhane K, Rappaport E B, Salam M T, Zhang Y and Gilliland F D 2014 Estimation of parameters in the two-compartment model for exhaled nitric oxide. PLoS ONE **9** e85471

Gelb A F, Taylor C F, Nussbaum E, Gutierrez C, Schein A, Shinar C M, Schein M J, Epstein J D and Zamel N 2004 Alveolar and airway sites of nitric oxide inflammation in treated asthma. American Journal of Respiratory & Critical Care Medicine **170** 737-741

George S C, Hogman M, Permutt S and Silkoff P E 2004 Modeling pulmonary nitric oxide exchange J.Appl.Physiol. **96** 831-839

Global Initiative for Asthma (GINA) 2018 Global Strategy for Asthma Management and Prevention https://ginasthma.org

Heijkenskjöld-Rentzhog C, Alving K, Kalm-Stephens P, Lundberg J O, Nordvall L and Malinovschi A 2012 The fraction of NO in exhaled air and estimates of alveolar NO in adolescents with asthma: Methodological aspects Pediatr.Pulmonol. **47** 941-949

Högman M, Holmkvist T, Wegener T, Emtner M, Andersson M, Hedenstrom H and Meriläinen P 2002 Extended NO analysis applied to patients with COPD, allergic asthma and allergic rhinitis Respir.Med. **96** 24-30

Högman M, Thornadtsson A, Liv P, Hua-Huy T, Dinh-Xuan A T, Tufvesson E, Dressel H, Janson C, Koskela K, Oksa P, Sauni R, Uitti J, Moilanen E and Lehtimäki L 2017 Effects of growth and aging on the reference values of pulmonary nitric oxide dynamics in healthy subjects J.Breath Res. **11** 047103-7163/aa7957

Horvath I, Barnes P J, Loukides S, Sterk P J, Högman M, Olin A, Amann A, Antus B, Baraldi E, Bikov A, Boots A W, Bos L D, Brinkman P, Bucca C, Carpagnano G E, Corradi M, Cristescu S, de Jongste J C, Dinh-Xuan A, Dompeling E, Fens N, Fowler S, Hohlfeld J M, Holz O, Jobsis Q, Van De Kant K, Knobel H H, Kostikas K, Lehtimäki L, Lundberg J, Montuschi P, Van Muylem A, Pennazza G, Reinhold P, Ricciardolo F L M, Rosias P, Santonico M, van der Schee M P, van Schooten F, Spanevello A, Tonia T and Vink T J 2017 A European Respiratory Society technical standard: exhaled biomarkers in lung disease European Respiratory Journal **49** 1600965

Kanazawa H, Kyoh S, Asai K and Hirata K 2010 Validity of measurement of two specific biomarkers for the assessment of small airways inflammation in asthma. Journal of Asthma **47** 400-406

Karvonen T, Kankaanranta H, Saarelainen S, Moilanen E and Lehtimäki L 2017 Comparison of feasibility and estimates of central and peripheral nitric oxide parameters by different mathematical models J.Breath Res. **11** 047102-7163/aa7cc0

Karvonen T and Lehtimäki L 2019 Flow-independent nitric oxide parameters in asthma: a systematic review and meta-analysis J.Breath Res. **13** 044001-7163/ab2c99

Lehtimäki L, Kankaanranta H, Saarelainen S, Hahtola P, Järvenpää R, Koivula T, Turjanmaa V and Moilanen E 2001 Extended exhaled NO measurement differentiates between alveolar and bronchial inflammation. American Journal of Respiratory & Critical Care Medicine **163** 1557-1561

Lehtimäki L, Karvonen T and Högman M 2020 Clinical Values of Nitric Oxide Parameters from the Respiratory System Curr.Med.Chem. **27** 7189-7199

María Rubio-Aparicio, José Antonio López-López, Wolfgang Viechtbauer, Fulgencio Marín-Martínez, Juan Botella and Julio Sánchez-Meca 2020 Testing Categorical Moderators in Mixed-Effects Meta-analysis in the Presence of Heteroscedasticity **88** 288-310

Molshatski N and Eckel S P 2017 Optimal flow rate sampling designs for studies with extended exhaled nitric oxide analysis. Journal of Breath Research **11** 016012

National Institute for Health and Clinical Excellence (NICE) 2017 Asthma: Diagnosis, Monitoring and Chronic Asthma Management NG80 https://nice.org.uk/guidance/ng80

Nihlberg K, Andersson-Sjoland A, Tufvesson E, Erjefalt J S, Bjermer L and Westergren-Thorsson G 2010 Altered matrix production in the distal airways of individuals with asthma. Thorax **65** 670-676

Paredi P, Kharitonov S A, Meah S, Barnes P J and Usmani O S 2014 A novel approach to partition central and peripheral airway nitric oxide. Chest **145** 113-119

Pedroletti C, Zetterquist W, Nordvall L and Alving K 2002 Evaluation of exhaled nitric oxide in schoolchildren at different exhalation flow rates Pediatr.Res. **52** 393-398

Pietropaoli A P, Perillo I B, Torres A, Perkins P T, Frasier L M, Utell M J, Frampton M W and Hyde R W 1999 Simultaneous measurement of nitric oxide production by conducting and alveolar airways of humans. J.Appl.Physiol. **87** 1532-1542

R Core Team 2016 *R: A language and environment for statistical computing. https://R-project.org/*

Robroeks C M, van Vliet D, Hendriks H J, Dompeling E and Jobsis Q 2010 Feasibility of exhaled nitric oxide measurements at various flow rates in children with asthma Pediatr.Allergy Immunol. **21** e222-8

Sardon O, Corcuera P, Aldasoro A, Korta J, Mintegui J, Emparanza J I and Perez-Yarza E G 2014 Alveolar nitric oxide and its role in pediatric asthma control assessment. BMC Pulmonary Medicine **14** 126

Shimoda T, Obase Y, Nagasaka Y, Nakano H, Kishikawa R and Iwanaga T 2016 Lung Sound Analysis and Airway Inflammation in Bronchial Asthma. The Journal of Allergy & Clinical Immunology in Practice **4** 505-511

Silkoff P, Sylvester J, Zamel N and Permutt S 2000 Airway nitric oxide diffusion in asthma - Role in pulmonary function and bronchial responsiveness American Journal of Respiratory and Critical Care Medicine **161** 1218-1228

Tsoukias N M and George S C 1998 A two-compartment model of pulmonary nitric oxide exchange dynamics. J.Appl.Physiol. **85** 653-666

Tsoukias N, Shin H, Wilson A and George S 2001 A single-breath technique with variable flow rate to characterize nitric oxide exchange dynamics in the lungs J.Appl.Physiol. **91** 477-487

Tufvesson E, Aronsson D, Ankerst J, George S C and Bjermer L 2007 Peripheral nitric oxide is increased in rhinitic patients with asthma compared to bronchial hyperresponsiveness Respir.Med. **101** 2321-2326

Viechtbauer W 2010 Conducting meta-analyses in R with the metafor package Journal of Statistical Software **36** 1–48

Wolfgang Viechtbauer 2020, *Comparing Estimates of Independent Meta-Analyses or Subgroups*, viewed 28 Nov 2020, <http://www.metafor-project.org/doku.php/tips:comp_two_independent_estimates>