Comparison of feasibility and estimates of central and peripheral nitric oxide parameters by different mathematical models

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ABSTRACT

Introduction: Assessing central and peripheral nitric oxide (NO) dynamics of the lung provides information on the severity and anatomical site of pulmonary inflammation. Several mathematical methods to calculate alveolar and bronchial NO parameters have been introduced. Our aim was to compare these methods.

Methods: The study included 69 healthy adults, 66 healthy children, 73 asbestos-exposed subjects and 72 subjects with chronic obstructive pulmonary disease (COPD). Exhaled NO was measured at multiple flow rates and we used five mathematical methods (Tsoukias & George, Pietropaoli, Condorelli, Högman & Meriläinen, and Silkoff) to estimate alveolar and bronchial NO parameters.

Results: H&M method was less frequently feasible compared to other methods but it had the highest degree of agreement with the measured data. The methods were most often feasible in healthy or asbestos-exposed adults but distinctly more infrequently in children and adults with COPD, suggesting difficulties in NO measurements in these groups. The linear methods (T&G, Pietropaoli) yielded higher alveolar NO concentration and lower bronchial NO flux than the two non-linear methods (H&M, Silkoff) and linear method with correction for axial back-diffusion of NO (Condorelli).

Conclusion: In differentiating central and peripheral NO sources we recommend using the linear methods, as low flow rates are not needed and the feasibility of the methods is good. If bronchial wall NO concentration ($C_{aw}NO$) and diffusing capacity ($D_{aw}NO$) are of interest, non-linear methods are needed and we recommend using H&M method as only three flow rates are needed. However, the agreement between the model and measured data needs to be checked in real-time to ensure feasibility. If the subject has difficulties with the extremely low or high flow rates, we then recommend using the Silkoff method to improve feasibility, but more flow rates and measurements are then needed and the agreement between the model and the measured data may be poorer.

1. Introduction

Measurement of exhaled NO concentration (F_ENO) is a promising tool to determine inflammation in different lung diseases. Currently, we have the best knowledge of asthma. In particular groups, F_ENO can be used to predict treatment responsiveness. For instance, a high F_ENO in asthma has been found to predict a good response to inhaled corticosteroids [1-3]. However, using more than one flow rate in NO measurement permits calculating bronchial and alveolar NO independently. This provides more information on the site of inflammation.

1.1. The Two-compartment model

Nikolaos M. Tsoukias and Steven C. George described the NO exchange dynamics of the lungs by the two-compartment model in 1998 [4]. In the two-compartment model, lungs are divided into two regions: expansible alveolar and rigid bronchial regions (Figure 1). Alveolar and bronchial regions are different in gas exchange perspective, enabling to calculate the origin of NO using the two-compartment model. The two-compartment model is capable of explain three experimentally observed features of the NO exchange dynamics: 1) holding breath prior to NO measurement creates a peak in F_ENO, 2) F_ENO is inversely proportional to flow rate, 3) NO elimination rate (V_{NO}) from the lungs is directly proportional to flow rate [5].

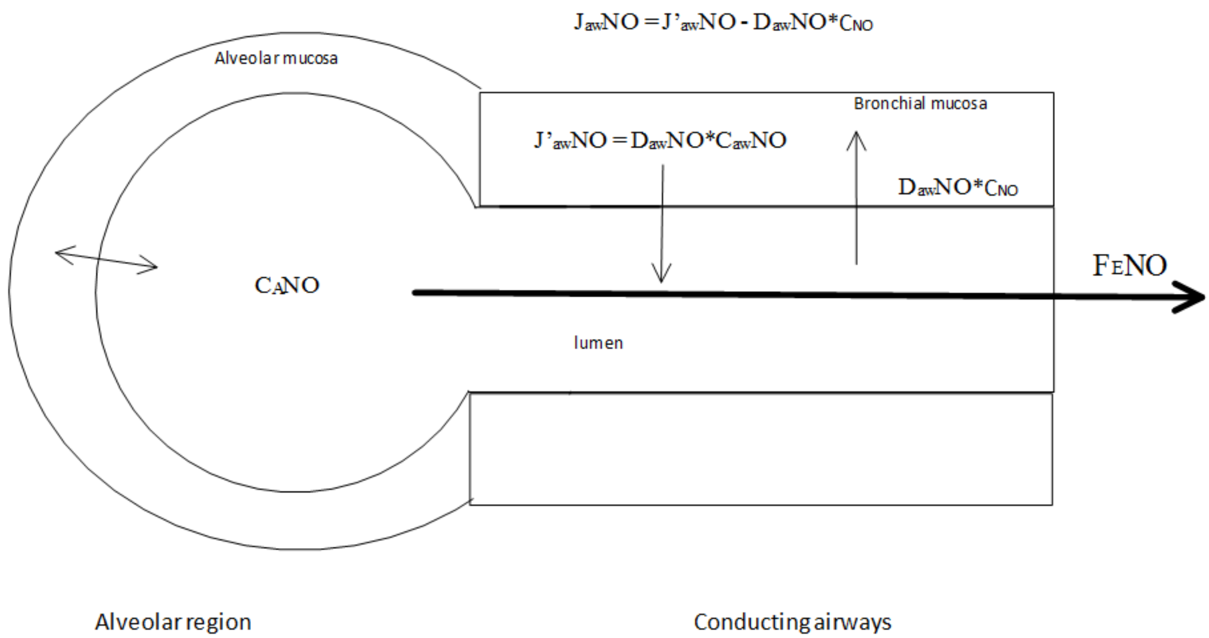

Figure 1. Drawing of the two-compartment model. Exhaled NO concentration (F_ENO) is the sum of alveolar and bronchial contributions. The bronchial or airway region's contribution depends on three flow-independent variables: maximum NO flux from the bronchial wall (J'_{aw}NO, pl/s), NO concentration of bronchial mucosa (C_{aw}NO, ppb) and diffusing capacity of NO from the bronchial wall (D_{aw}NO, pl/s/ppb). Alveolar contribution depends on steady state alveolar NO concentration (C_ANO, ppb).

1.2. The Trumpet model with axial back-diffusion (TMAD)

The two-compartment model assumes the conducting airways as an even, cylinder shaped tube, neglecting the fact that the bronchial tree branches towards the more distal airways. Condorelli et al. introduced the trumpet model of the lungs with correction for axial back-diffusion of NO (TMAD), where the conducting airways are considered as trumpet shaped [6]. This takes account the increasing total cross-sectional area and the mucosal surface area of the airways as the bronchial tree branches into smaller bronchioles. As the total cross-sectional area of the airways increases, the velocity of airflow decreases, especially in the last generations of the bronchial tree. The significant decrease in airflow velocity allows axial back-diffusion of NO from the higher NO concentration of the conducting region into the respiratory region, according to the NO concentration gradient. It has been proposed that neglecting this axial back-diffusion of NO leads to over estimation of C_ANO and under estimation of J_{aw}NO. However, this model is recommended to be utilized only in subjects with no obstruction or it can lead to overcorrection [7,8]. Some studies have also reported that the TMAD-correction leads to negative C_ANO values [9-11].

1.3. The flow-independent NO parameters

The two-compartment model describes alveolar and bronchial regions' NO exchange dynamics by flow-independent NO parameters: C_ANO (steady state NO concentration in the alveolar region) describing the alveolar region, $C_{aw}NO$ (NO concentration in bronchial mucosa) and $D_{aw}NO$ (NO diffusing capacity of the bronchial wall) representing the bronchial region. Maximal bronchial NO flux can be calculated as $J'_{aw}NO = D_{aw}NO * C_{aw}NO$ and bronchial NO flux as $J_{aw}NO = D_{aw}NO (C_{aw}NO - C_ANO)$. Once flow-independent parameters are determined, the two-compartment model can be used to predict exhaled NO concentration (ppb) in certain exhalation flow rate (V_E) by exponential function

$$F_ENO = C_{aw}NO + (C_ANO – C_{aw}NO) * \exp(-D_{aw}NO/V_E) \quad [4,5,12,13] \tag{1}$$

When V_E is large compared to $D_{aw}NO$ ($V_E > \sim 5* D_{aw}NO$), the exponential function approaches its first-order linear approximation ($\exp(-D_{aw}NO/Ve) = 1 – D_{aw}NO/V_E$) [5]. When this approximation is used in equation 1, the equation reduces to following

$$F_ENO = C_ANO + (C_{aw}NO – C_ANO)D_{aw}NO*\frac{1}{\mathrm{VE}} \tag{2}$$

Knowing the fact that $J_{aw}NO = D_{aw}NO(C_{aw}NO - C_ANO)$, equation 2 can be reduced to

$$F_ENO = C_ANO + J_{aw}NO*\frac{1}{\mathrm{VE}} \tag{3}$$

When both sides of this equation 3 are multiplied by V_E, linear equation of NO elimination rate is derived

$$V_{NO} = C_ANO * Ve + J_{aw}NO \tag{4}$$

1.4. Analytical methods

There are several techniques to approximate the flow-independent NO parameters based on measurements of F_ENO at multiple flow rates. In this study, we used four different methods which will be introduced herein. Tsoukias et al. [14] used linear equation 4 above in assessing C_ANO and $J_{aw}NO$ (Figure 2A). In this method, F_ENO is measured at several high (≥100 ml/s to verify that $V_E > \sim 5* D_{aw}NO$) flow rates. V_{NO} is calculated from measured F_ENO and the flow rates used. Then V_{NO} is plotted against flow rate V_E and a regression line is set between V_{NO} and V_E. C_ANO is obtained from the slope and $J_{aw}NO$ from the intercept of the regression line.

Pietropaoli et al. [12] utilized linear equation 3 to estimate C_ANO and $J_{aw}NO$ (figure 2B). In this method, the measured value of F_ENO at several high (≥100 ml/s) flow rates are plotted against $\frac{1}{VE}$ and a regression line is set between F_ENO and $\frac{1}{VE}$. The intercept is used as an estimate for C_ANO whereas slope for $J_{aw}NO$.

Condorelli et al. used the Tsoukias & George method to calculate C_ANO and $J_{aw}NO$ with correction of the axial back-diffusion by applying correction factors a and b in the following equations [6]:

$$C_ANO^{Condorelli} = C_ANO^{T\&G} - * \frac{1}{a} J_{aw}NO^{T\&G} \quad (5)$$

$$J_{aw}NO^{Condorelli} = J_{aw}NO^{T\&G} * b \quad (6)$$

The values of correction factors a and b are dependent on flow rates used in measurements.

Silkoff et al. [15] utilized a nonlinear regression analysis using equation 1 to determine all three flow-independent parameters by measuring F_ENO at several high and low flows (figure 2C). Högman et al. [13,16] used a method that combines some of the methods described above (figure 2D). Three flow rates are needed: one low, one medium and one high. C_ANO is estimated as in Tsoukias & George method: a regression line is set between V_{NO} and V_E at medium and high flow rates, and the slope is used as an estimate of C_ANO. $D_{aw}NO$ is obtained using low and medium flow rates with an iterative technique, whereas $C_{aw}NO$ is estimated using $D_{aw}NO$ and C_ANO obtained earlier.

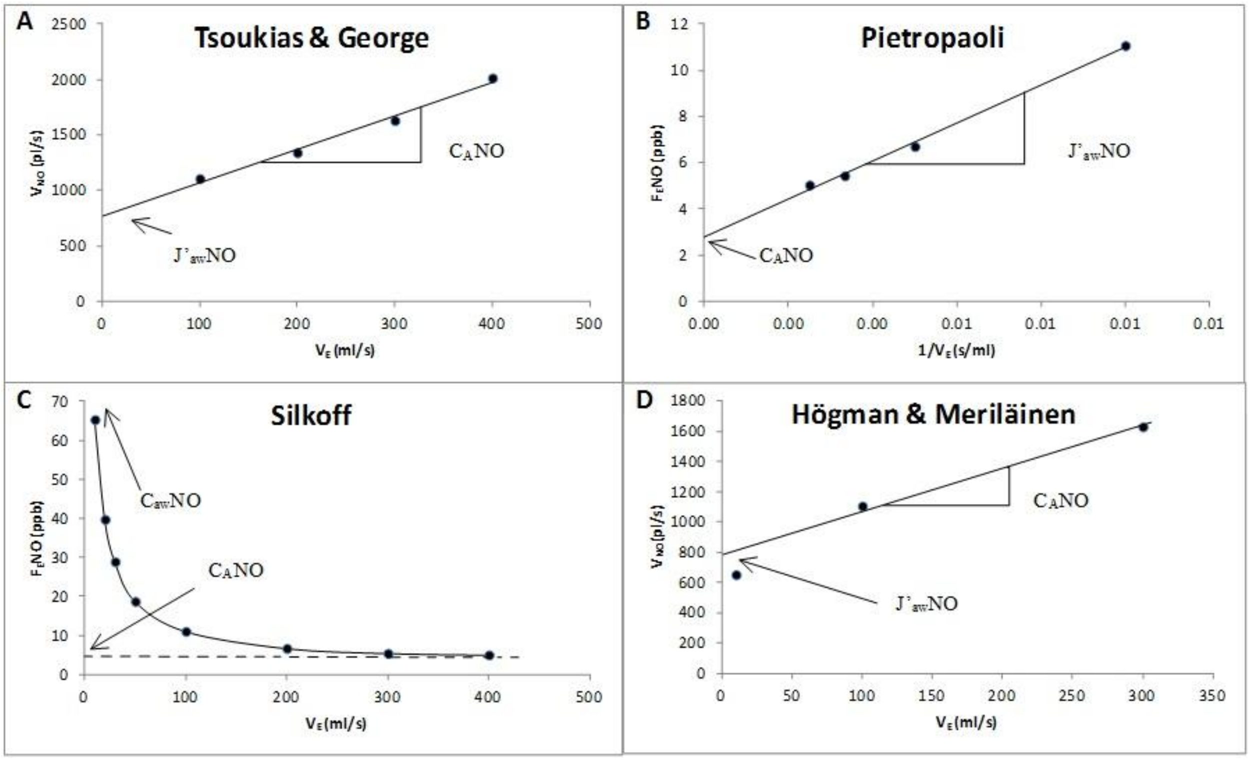

Figure 2. Schematics describing the mathematical methods used. Tsoukias & George A: V_{NO} is plotted against flow rate V_E and a regression line is set between V_{NO} and V_E. C_ANO is obtained from the slope and $J_{aw}NO$ from the intercept of the regression line. In Condorelli method, parameters are calculated likewise before applying the flow rate dependent correction factors. Pietropaoli B: F_ENO is plotted against $\frac{1}{\mathrm{VE}}$ and a regression line is set between F_ENO and $\frac{1}{\mathrm{VE}}$. The intercept is used as an estimate for C_ANO whereas slope for $J_{aw}NO$. Silkoff C: F_ENO is plotted against V_E using all measured flow rates. $C_{aw}NO$ is the intercept of the plot. C_ANO is the limit of F_ENO when V_E approaches infinity ($F_ENO = C_ANO$, when $V_E \rightarrow \infty$). $D_{aw}NO$ describes the steepness of the plot: small $D_{aw}NO$ makes the plot steeper. The parameters are calculated by fitting the governing equation (Equation 1) to the observed data by regression analysis. Högman & Meriläinen D: C_ANO and $J_{aw}NO$ are obtained by using high and medium flow rates as in Tsoukias & George method. $D_{aw}NO$ is then calculated using $J_{aw}NO$ and F_ENO at low and medium flow rates using an iterative technique. $C_{aw}NO$ is then calculated by using all previously calculated parameters.

1.5. Aims of the study

It is important to know whether there are significant differences between the existing methods in NO parameter estimation. Sometimes the original measurement data does not fit the mathematical equation used to estimate the flow-independent NO parameters and the iterative process does not converge or impossible results are obtained (i.e. the method is not feasible in that subject). There may also be differences in the model´s degree of agreement with the measured data (i.e. what is the difference between measured F_ENO and calculated F_ENO). Also, the magnitudes of calculated NO parameters may differ between methods and used flow rates. In spite of importance in the development of the methods, comparative studies are scarce.

The aim of the present study was to compare different analytical methods (Tsoukias & George (T&G), Pietropaoli, Condorelli, Silkoff and Högman & Meriläinen (H&M)) to calculate NO parameters regarding 1) their feasibility (how often the mathematical analysis succeeded and provided results that were not considered as outliers or physically impossible), 2) their degree of agreement with measured data (difference between calculated and observed F_ENO value), 3) the magnitude of calculated NO parameters and 4) the effect of the used flow rates on the feasibility and calculated NO parameters.

2. Methods

2.1. Subjects

In the analysis, we included subjects from several of our previously published studies utilizing F_ENO measurements at multiple flow rates. The groups were 69 healthy adults, 66 healthy children, 72 adults with COPD and 73 adult subjects with previous asbestos exposure. The inclusion and exclusion criteria and clinical characteristics are previously published [17-19]. In short, subjects with COPD were newly diagnosed with no current anti-inflammatory treatment. They had symptoms compatible with COPD, post-bronchodilator FEV1/forced vital capacity (FVC) ratio < 0.7, smoking history of at least 20 pack-years, and emphysema on high-resolution computed tomography (HRCT) of the lungs. The subjects with previous asbestos exposure were non-smoking males with a history of moderate to severe occupational exposure to asbestos without other known respiratory diseases.

As the sample size in the current study is predetermined by the original studies, we made a post hoc calculation of statistical power for the current analysis. We considered differences in NO parameters given by the 5 different calculation methods as main outcome. Using repeated measures ANOVA with α-error of 5 %, a sample size of 31 subjects is needed to get statistical power of 95 % to detect a difference between the methods that is 0.25 times standard deviation (to keep the estimate conservative we estimated correlation among repeated measures to be 0.5 and used correction for nonsphericity as 1, G*Power 3.1.7 was used in calculations). As nonparametric tests are usually more conservative that parametric tests and as we used nonparametric Friedman’s test instead of parametric ANOVA in comparisons, we consider that we have sufficient statistical power in each comparison where the number of subjects with successful measurements is above forty.

2.2. NO measurement

As previously described [17-19], exhaled NO was measured using Sievers NOA 280 chemiluminescent analyzer (Sievers Instruments, Boulder, Colorado, USA) at different flow rates. We outlaid the flow rates of 10, 50, 100, 200 and 300 ml/s for the healthy children, whereas flow rates of 10, 20, 30, 50, 100, 200, 300 and 400 ml/s were used for the adult subjects. At least two successful NO measurements with coefficient of variation (CV) of maximum 3 % were performed at each flow rate. The desired flow rates were achieved by letting the subjects exhale through a mass flow meter connected to a computer-controlled, adjustable flow restrictor that kept the flow rate steady at the desired level. The linearity of V_{NO} at flows ≥ 100 ml/s was evaluated later in the analysis phase and r < 0.95 was used as an exclusion criterion in the linear methods.

2.3. Calculation of NO parameters

Before performing any calculations, we visually inspected every F_ENO versus flow rate plot. Plots were accommodated by omitting failed measurements (i.e. if F_ENO at 300 ml/s < F_ENO at 400 ml/s, flow rate of 400 ml/s can be discarded as failed measurement) and using nearest successful measurement point if possible. In healthy adults, the flow rate of 400 ml/s was excluded 14 times and at least one of the flow rates between 10 and 30 ml/s were excluded 4 times. The corresponding numbers for previous asbestos exposure group were 9 and 3 and in the group of COPD patients 13 and 4. Only 2 300 ml/s flow rates were excluded in healthy children. If the plot was beyond accommodation, the subject was excluded from the analysis. A total of 15 plots were beyond accommodation in COPD patients and 2 in healthy children in linear methods (these were considered as visual drop-outs). The failed flow rates were almost exclusively 300 ml/s and 400 ml/s. Five different analytical methods were used (T&G, Pietropaoli, Condorelli, Silkoff and H&M) to calculate the NO parameters in every subject group (Figure 2). All calculations were performed by using Microsoft Excel 2007.

2.3.1 Linear methods

In T&G method, V_{NO} was plotted against V_E and we used Excel functions “slope” and “intercept” to calculate C_ANO and $J_{aw}NO$, respectively. Exhalation flow rates of 100, 200, 300 and 400 ml/s were used for adults, whereas 100, 200, 300 ml/s were used for children. Correlation coefficient between flow rate and V_{NO} was calculated by using the correlation function in Excel. In Pietropaoli method, we used the same flow rates as in T&G method. F_ENO was plotted against $\frac{1}{VE}$ and C_ANO was calculated using the intercept function and $J_{aw}NO$ with the slope. The correlation coefficient between $\frac{1}{VE}$ and F_ENO was also calculated.

In Condorelli method, NO parameter estimates of the T&G method were used. Correction for axial back-diffusion was then achieved by using the correction factors (a= 1100 ml/s, b=1.6 with flow rates 100 – 400 ml/s and a= 840 ml/s, b=1.7 with flow rates 100 – 300 ml/s) as introduced earlier (Equations 5 and 6). Failed measurements were excluded from the calculations as previously described. In adult subjects, one failed flow rate was allowed to be excluded. In the case of more than one failed flow rate, we excluded the subject.

2.3.2 Non-linear methods

In H&M method, we used an Excel calculation sheet made by Pekka Meriläinen, one of the inventors of the method. We compared the results using different three flow rate combinations. The best combination of flow rates was found to be 10, 100 and 300 ml/s as it yielded relevant results with least outliers. Failed measurements were optimized by using the nearest possible flow rate in place of a failed one (e.g. replacing a failed 10 ml/s by 20 ml/s or failed 400 ml/s by 300 ml/s).

In Silkoff method we compared Solver tool in Microsoft Excel 2007 and a commercial regression analysis software NLREG (nlreg.com). However, as both were found to function equally well in our purpose, we chose the Excel Solver to be used in the analysis. The Silkoff method is based on non-linear regression analysis in which observational data are modeled by a non-linear function. The calculated plot is attempted to fit the observational data by changing the model's parameters. We performed the non-linear regression analysis by using the least squares algorithm. The sums of the squared residuals of the observed and calculated F_ENO values at different flow rates are minimized by changing the model's parameters ($D_{aw}NO$, $C_{aw}NO$ and C_ANO).

This method requires initial starting value for the iteration process. We used the medians of the C_ANO, $C_{aw}NO$ and $D_{aw}NO$ calculated for healthy adults using the H&M method as an initial starting value. After the iterations, the calculated parameter values were set as the new starting values for a new iteration process. The iteration cycle was repeated three more times, setting each time the previously obtained parameter values as new starting values. The number of iterations within each cycle depended on how quickly the Excel Solver converged to a solution with all constrains satisfied (precision = 0.000001, tolerance 5% and convergence 0.0001). We used all available successful flow rates in calculations.

2.4. Analysis

2.4.1. Checking the feasibility and excluding outliers

After the calculation of the NO parameters with each of the methods, we evaluated the methods' feasibility in parameter estimation and excluded the outliers using the following criteria $C_ANO < 0$, $C_ANO > 10$, $C_{aw}NO < 0$ or $C_{aw}NO > 1000$. The upper limits were set according to previous results [15,17,20-23] and histograms and lower limits are based on the fact that negative concentrations are not physically possible. Defining outlier criteria for $D_{aw}NO$ was not considered necessary as excluding abnormal C_ANO and $C_{aw}NO$ values excluded abnormal $D_{aw}NO$ and $J_{aw}NO$ values as well at the same. This is explained by the fact that $C_{aw}NO$ and $D_{aw}NO$ are mathematically coupled and in cases of extremely low CawNO, DawNO is extremely high, and vice versa. In the linear methods, correlation coefficient less than 0.95 was also used as an exclusion criterion. In H&M method there is an internal validity check also included. Negative, or otherwise impossible results as well as inferior correlation may conclude that the model of NO production of the respiratory system may be inadequate. It may also be due to problems in the measurement situation. A Cochran´s Q test was used to assess if the proportion of feasibility differed significantly between the methods. If the p-value with Cochran's Q test was < 0.05, pairwise comparison was performed to check which methods differed significantly from one another. IBM SPSS Statistics 24 was used in the statistical analysis.

2.4.2. Checking the method's degree of agreement.

We evaluated the methods' degree of agreement with the observational data by calculating absolute values of the difference between observed and calculated values of F_ENO. A flow rate of 100 ml/s was used in the comparison of the measured and calculated F_ENO, since linear methods apply with higher flow rates only and this flow rate also did not produce difficulties for most of the subjects. The difference between the methods was compared using nonparametric Friedman's repeated two-way ANOVA.

2.4.3. Comparing the magnitude of NO parameters between the methods.

After calculating all the NO parameters with all the methods, the distributions of the calculated parameters were compared between different methods using nonparametric Friedman's repeated two-way ANOVA. The results are presented as median, inter-quartile range (IQR), minimum and maximum in the tables.

2.4.4. The effect of the used flow rates on the feasibility and NO parameters.

We also assessed the effect of used flow rates in parameter estimates. We calculated the NO parameters for healthy adults with all flow rates successful (n = 56) using two different methods, linear T&G and non-linear H&M. Flow rates of 100, 200 and 300 ml/s; 200, 300 and 400 ml/s; and 100, 200, 300 and 400 ml/s were used in T&G method, whereas flow rates of 10, 100 and 400 ml/s; 10, 100 and 300 ml/s; 20, 100 and 400 ml/s; and 20, 100 and 300 ml/s were used in H&M method, respectively. After calculating the parameters, outliers were excluded as previously described. The distributions of the calculated parameters were compared between different flow rates using nonparametric Friedman's repeated two-way ANOVA.

3. Results

Basic demographics of the subjects are introduced in the Table 1.

Table 1. Basic demographics of the subjects.

	Healthy adults	Healthy children	Asbestos exposure	COPD
N	69	66	73	72
Sex	61 / 8	32 / 34	All males	50 / 22
Age	63 (63.5 ± 7.4)	9 (9.7 ± 1.6)	65 (64.9 ± 6.6)	58 (58.5 ± 7.6)
Fev1 % pred.	n.a.	n.a.	89.0 (88.3 ± 14.0)	52.0 (53.8 ± 14.81)

Sex is presented as (males / females)
Age and Fev1 % pred. are presented as median (mean ± SD)

3.1. Feasibility of the methods

The median correlation coefficients for linearity of V_{NO} against flow at flow rates ≥ 100 ml/s were > 0.99 in all groups, indicating a good linearity of measurements. We discovered significant differences in the feasibility of different methods between subject groups and between the methods (Table 2). Of the different subject groups, the feasibility of all methods was best in healthy adults and in adults with asbestos exposure. In healthy children, the feasibility was poorer and it was poorest in subjects with COPD.

When comparing the different methods in each subject group, linear methods tended to yield results slightly more often compared to the non-linear ones. In subject groups where the feasibility was lower (children and subjects with COPD), H&M method was feasible significantly less frequently than the other methods.

Table 2. Comparison of the feasibility of the mathematical methods in the parameter estimation and reasons for excluding outliers.

	T&G	Pietropaoli	Condorelli	Silkoff	H&M
Healthy Adults					
Total number of subjects	69	69	69	69	69
Visual estimation drop-out	0	0	0	0	0
Outliers excluded, total	6	2	8	8	11
$C_ANO < 0$			2	4	
$C_ANO > 10$					
$C_{aw}NO > 1000$				4	9
r < 0.95 or H&M validity check	6	2	6		2
Successful results	63*	67	61	61	58
Healthy children					
Total number of subjects	66	66	66	66	66
Visual estimation drop-out	2	2	2	1	1
Outliers excluded, total	2	1	3	8	17
$C_ANO < 0$			1	1	
$C_ANO > 10$					
$C_{aw}NO > 1000$				7	16
r < 0.95 or H&M validity check	2	1	2		1
Successful results	$62^{H\&M}$	$63^{H\&M}$	$61^{H\&M}$	$57^{H\&M}$	48
Asbestos exposure					
Total number of subjects	73	73	73	73	73
Visual estimation drop-out	0	0	0	0	0
Outliers excluded, total	2	0	3	4	7
$C_ANO < 0$			1		
$C_ANO > 10$					
$C_{aw}NO > 1000$				4	7
r < 0.95 or H&M validity check	2		2		
Successful results	71**	73	70	69	66
COPD					
Total number of subjects	72	72	72	72	72
Visual estimation drop-out	15	15	15	0	0
Outliers excluded, total	6	13	6	17	49
$C_ANO < 0$			1	3	
$C_ANO > 10$	4	3	3		
$C_{aw}NO > 1000$				14	37
r < 0.95 or H&M validity check	2	10	2		12
Successful results	$51^{H\&M}$	$44^{H\&M}$	$51^{H\&M}$	$55^{H\&M}$	23

* Overall p-value 0.097, **Overall p-value 0.067, $^{H\&M}$ Significant difference against H&M method ($p < 0.05$)

3.2. Methods' degree of agreement with measured data

We evaluated the degree of agreement of the analytical methods with the measured data by calculating the absolute values of the differences between measured and calculated F_ENO values at flow rate 100 ml/s (Supplementary Table 1). Overall among the linear methods, Condorelli method tended to have a better degree of agreement with observational data than T&G from which it is derived as it yielded smaller residuals of observed and calculated F_ENO values. Pietropaoli method seemed to have a better agreement in every group except the healthy children than other linear methods. Condorelli method had the same degree of agreement as Silkoff in children and COPD patients, whereas Silkoff and T&G methods gave similar residuals to each other in every group. H&M and Pietropaoli seemed to have greater degree of agreement with the observational data in all groups except the healthy children. The non-linear methods differed from each other in every group. Overall, H&M method appeared to have the greatest agreement with observational data.

3.3. Comparing the magnitude of NO parameters between the methods

We discovered statistically significant differences among the results of the compared analytical methods (Figures 3-6 and Supplementary tables 2-5). Generally, there were no significant differences between T&G and Pietropaoli. However as might be expected, Condorelli method with axial back-diffusion correction yielded lower estimates for C_ANO and higher estimates for $J_{aw}NO$. Interestingly, Silkoff and Condorelli methods produced similar C_ANO estimates with no significant differences in any group. The other linear methods tended to give higher estimates for C_ANO and lower estimates for $J_{aw}NO$ as compared to non-linear methods, but the absolute difference was quite small and the differences were not all significant. H&M and Pietropaoli methods also yielded similar results occasionally. When comparing the non-linear methods to each other, Silkoff method gave higher estimates of $D_{aw}NO$ and lower estimates of $C_{aw}NO$ as compared to H&M. There were no consistent differences in C_ANO and $J_{aw}NO$ between H&M and Silkoff methods.

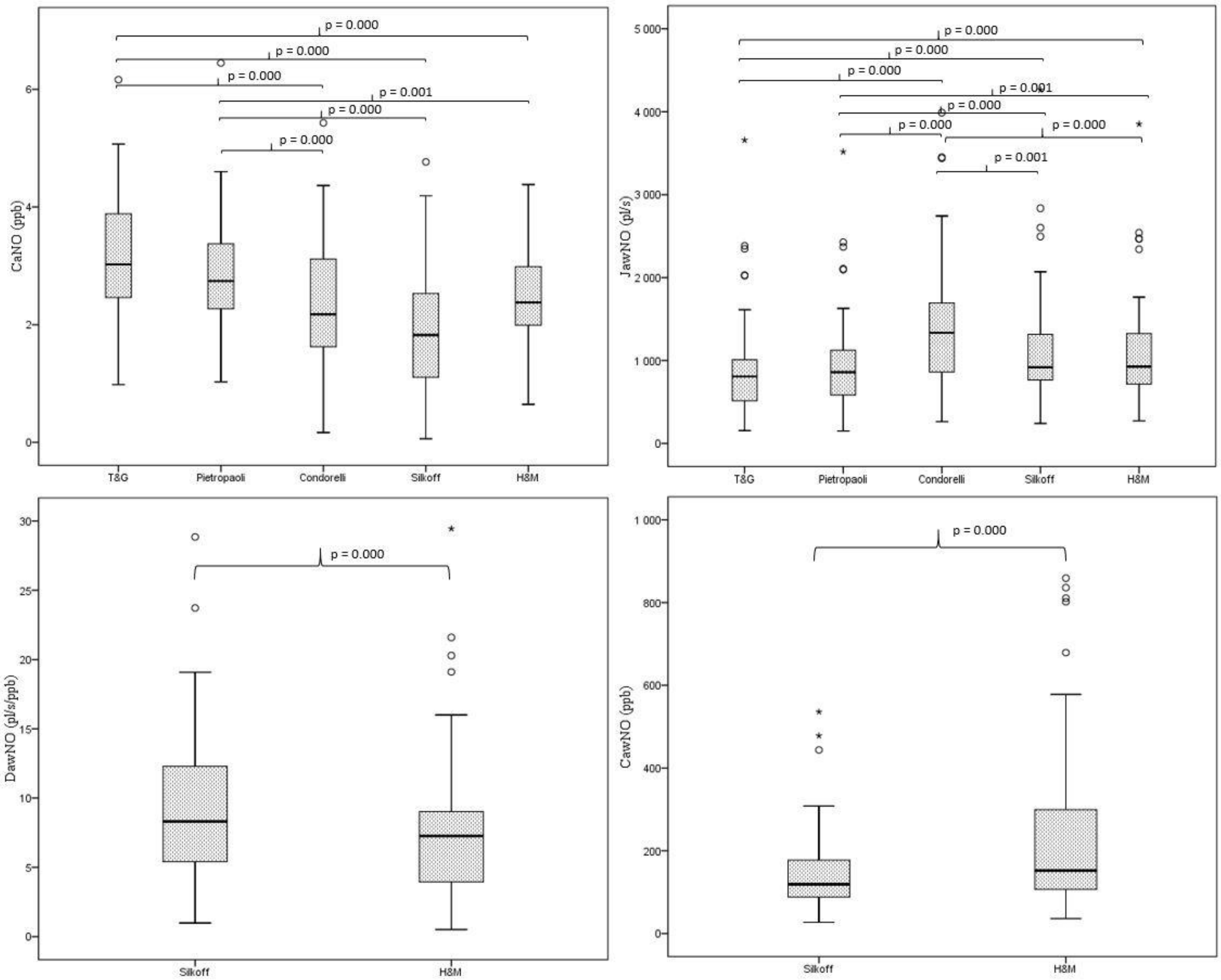

Figure 3. Box plot presentation of the NO parameters of healthy adults using different analytical methods. Only statistically significant differences are marked.

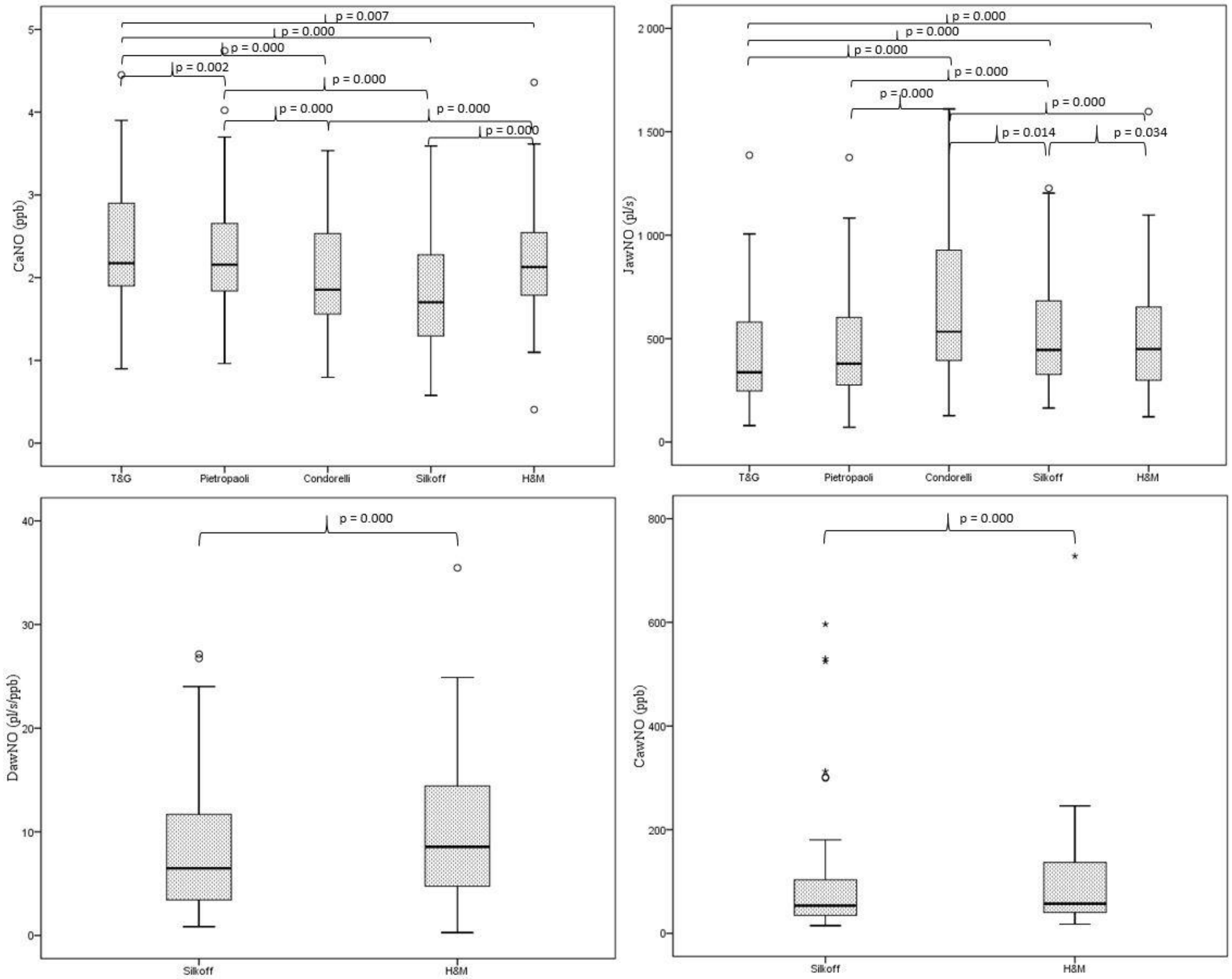

Figure 4. Box plot presentation of the NO parameters of healthy children using different analytical methods. Only statistically significant differences are marked.

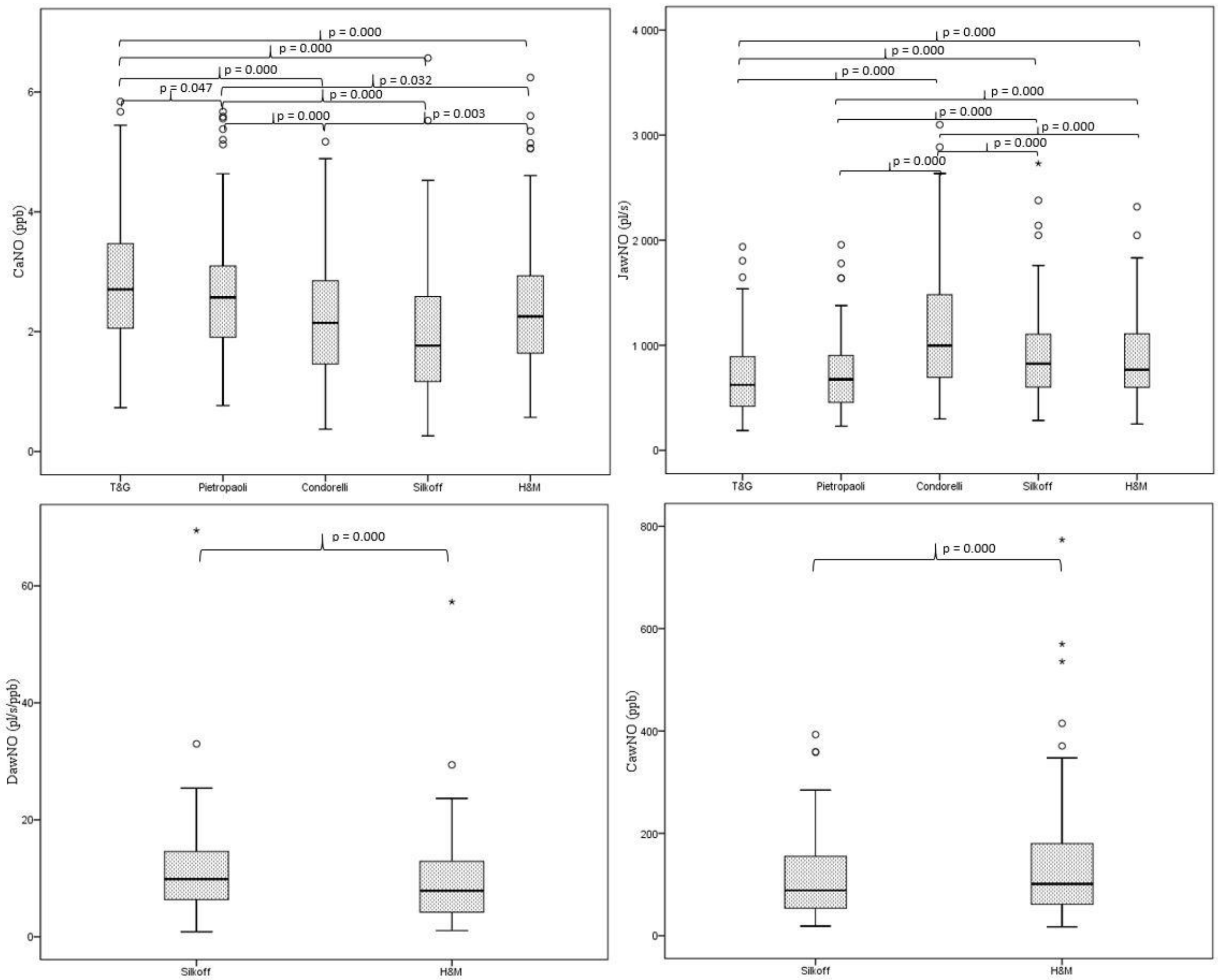

Figure 5. Box plot presentation of the NO parameters of asbestos exposed subjects using different analytical methods. Only statistically significant differences are marked.

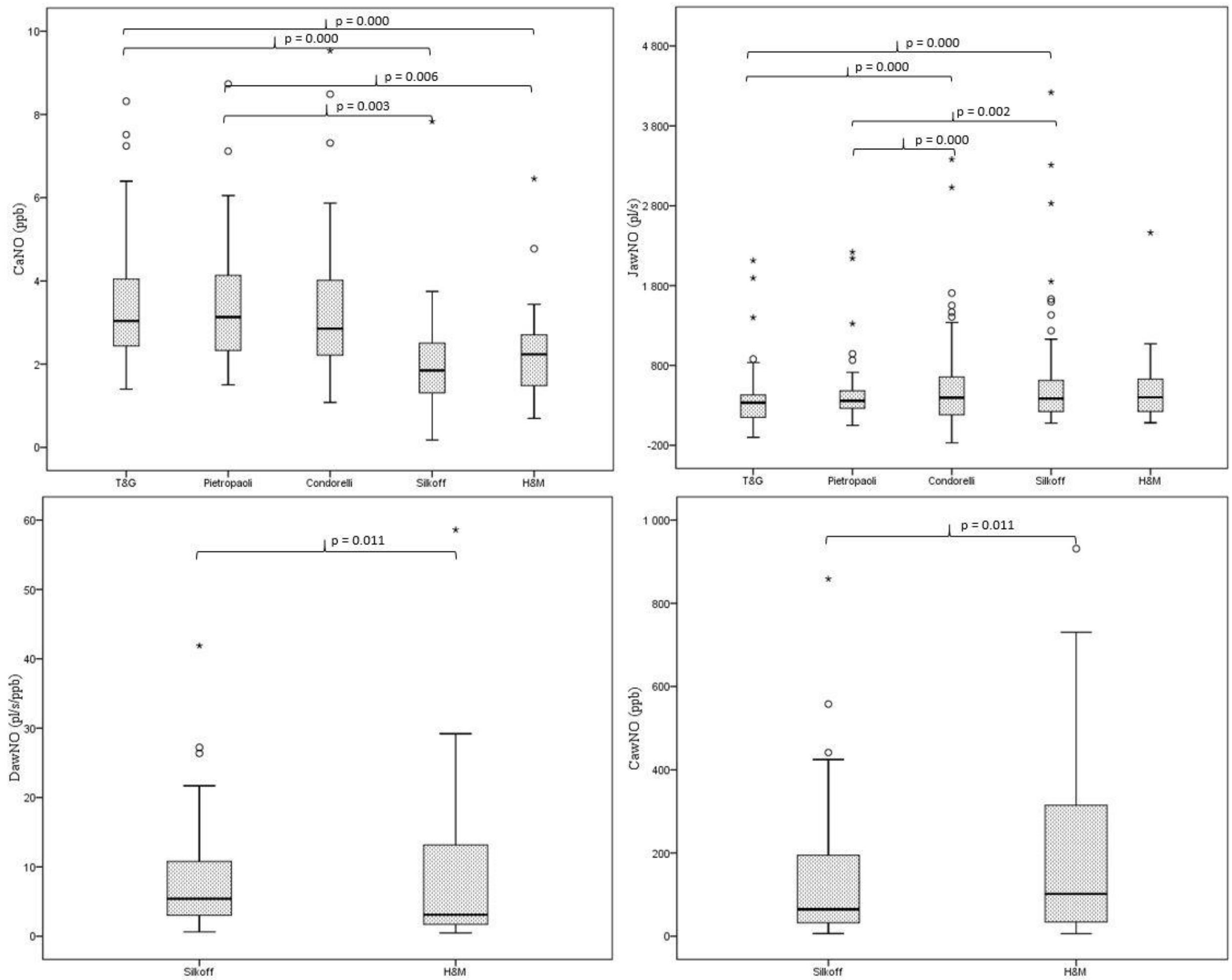

Figure 6. Box plot presentation of the NO parameters of COPD patients using different analytical methods. Only statistically significant differences are marked.

3.4. Comparing the effect of using different flow rates on feasibility and NO parameters

Different flow rates were compared in healthy adults using T&G and H&M (Figures 7 and 8) and statistically significant differences were observed in the parameters. In T&G, there were no differences in feasibility between flow rates, but difference was found between all flow rate combinations in both parameter values. Correlation coefficients had essentially no differences between different flow rate combinations, possibly due to exclusion of all failed measurements.

In the H&M method flow rates of 20, 100, 300 ml/s and 20, 100, 400 ml/s differed among every parameter, whereas flow rates of 10, 100, 400 ml/s and 20, 100, 300 ml/s were the same among every parameter. Differences in feasibility were found between flow rates 10, 100, 400 ml/s and 20, 100, 400 ml/s (44 vs 33 successful results, p = 0.008) and between flow rates 20, 100, 400 ml/s and 10, 100, 300 ml/s (33 vs 48 successful results, p = 0.000).

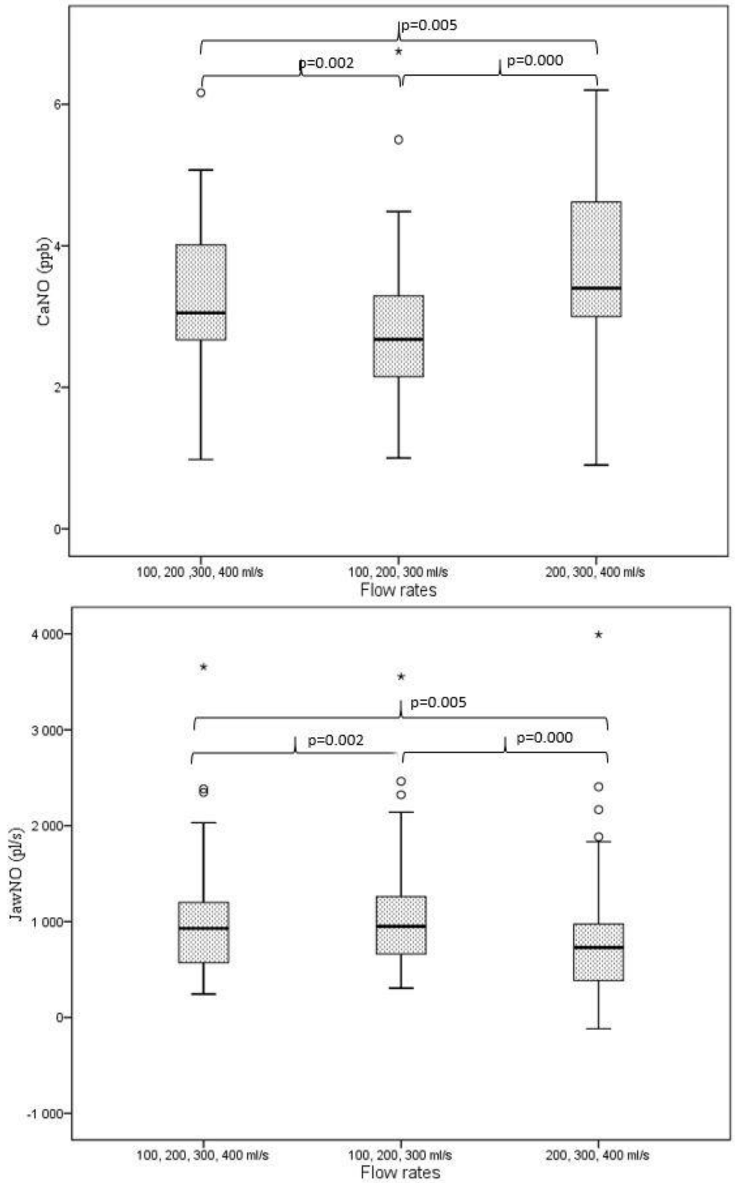

Figure 7. Box plots of the healthy adults' NO parameters using different flow rates in T&G method.

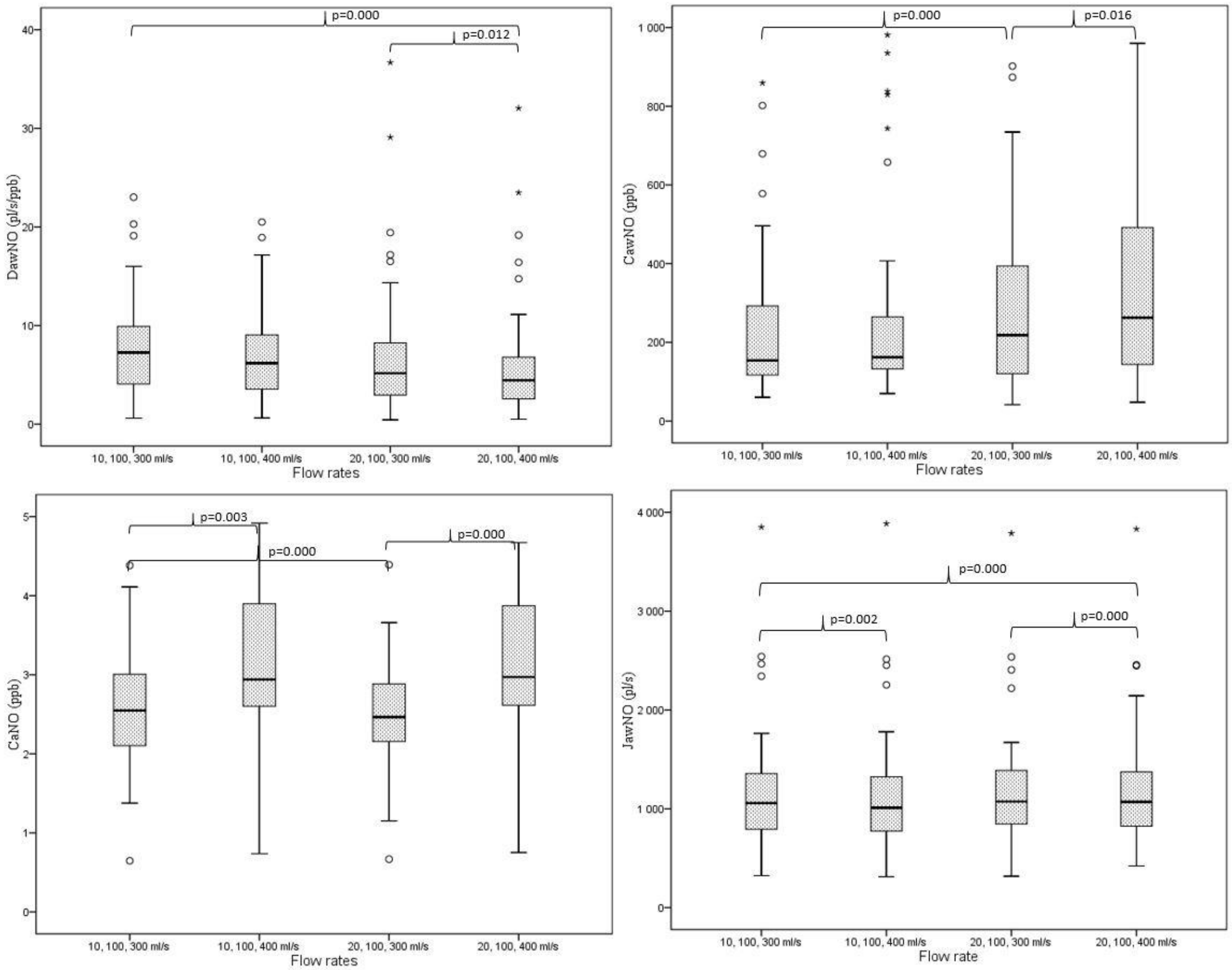

Figure 8. Box plots of the healthy adults' NO parameters using different flow rates in H&M method. Only statistically significant differences are marked.

4. Discussion

The feasibility of the four methods to calculate NO parameters and their agreement with measured data varied between the methods and between the subject groups. Linear methods and Silkoff method were most frequently feasible, whereas H&M had the lowest level of feasibility. However, when feasible, H&M had the highest degree of agreement with the data followed by the linear methods (T&G, Pietropaoli and Condorelli). The methods were most often feasible in healthy or asbestos-exposed adults but distinctly more infrequently in children and adults with COPD. There were also differences in the calculated NO parameters between the four methods but the two linear methods (T&G, Pietropaoli) yielded quite similar results as did the two non-linear methods (H&M, Silkoff). Condorelli method yielded smaller values for C_ANO and larger values for J_{aw}NO than other linear methods, as expected, and the C_ANO estimates were essentially the same between Silkoff and Condorlli methods. There is very little previous data comparing these mathematical methods in their feasibility and agreement with measured values.

4.1. Clinical relevancy and current standardization of multiple flow NO measurements

F_ENO measurement at multiple flow rates has several promising clinical applications. For instance, it has been noticed to be able to detect inflammation in the smaller airways in asthma [24-26]. Peripheral NO production (C_ANO) may also be useful in detecting other inflammatory diseases such as alveolitis and cystic fibrosis [19,20,26-28]. Currently, the clinical relevancy of C_{aw}NO and D_{aw}NO is pending. Thus, the clinical importance of non-linear methods over the linear methods is unclear at the moment, although they provide interesting information for research purposes. Potential use for C_{aw}NO and D_{aw}NO might be for example to tell whether elevated F_ENO is due to inflammation in the airways or change in airway diffusivity of nitric oxide [29,30]. D_{aw}NO and C_{aw}NO might therefore be used in airway diseases as separate measures of tissue remodeling and inflammatory activity, respectively. Clinical relevancy of all the NO parameters is unknown especially in COPD patients, where different pathophysiological aspects of the disease, such as emphysema, may have opposing effects on NO parameters. Also, the parameters should be interpreted cautiously as the current models do not take account the possible effect of obstruction. It has been reported that obstruction may lead to over correction in Condorelli method (lower C_ANO and higher J_{aw}NO values are obtained) [7,8].

Currently the predominant problem in F_ENO measurement at several flow rates is the lack of technical standardization. Different studies have used several different analyzers, setups, flow rates and mathematical methods, making the comparison of the NO parameters between studies challenging. NO parameters are known to vary with different used flow rates [31,32] and it is not known whether results obtained using different analyzers yield different estimates for NO-parameters as research has only been done on F_ENO values using single flow rates [33-39]. A recent ERS technical guideline [40] has now for first time given recommendations also on the multiple flow rate NO measurements, but many aspects are still not validated and not standardized. For instance, repeatability of NO parameters, possible circadian variation and differences between distinct NO analyzers require further research.

4.2. Feasibility of the methods and their agreement with measured data

The linear methods were more often feasible (i.e. the mathematical analysis succeeded and provided results that were not considered as outliers) than the non-linear methods. This may be related to more simple mathematics and the avoidance of extremely low flow rates that are difficult to some subjects. The drawback of linear methods is that neither $D_{aw}NO$ nor $C_{aw}NO$ can be estimated. However, these parameters have a minor clinical significance at the moment but may prove to be important when more knowledge on their clinical meaning is gained. The difference between the linear methods was not consistent in feasibility but the Pietropaoli method had on average better agreement with the data.

We found that among the non-linear methods Silkoff method was more often feasible than H&M method. H&M method's strength is that only three flow rates are needed, but on the other hand, this makes the method also more vulnerable for measurement errors at any single flow rate. However, H&M method had the best agreement with our observational data, suggesting that when it is feasible then the quality of data is high and that the better feasibility of the Silkoff method comes with the price of lower agreement with measured data in addition to the need for more flow rates.

Clearly the feasibility of all methods was the poorest in children and especially in subjects with COPD. This is probably related to the subjects' ability to perform reliable F_ENO measurements at very low and very high flow rates that usually are the most difficult ones. Molshatski et al. found that high flow rates were most important for estimating C_ANO, while low flow rates were especially important for estimating $D_{aw}NO$ [31]. This finding may explain the fact that in our study H&M method yielded most relevant results using 10 ml/s as the lowest flow rate, as recommended by Högman and colleagues [13,16]. The extreme flow rates appeared to be problematic especially in the group of COPD patients, explaining the inferior feasibility of linear methods and H&M method in that group.

Checking for the agreement between the used model and measured data in real-time might help to ensure the quality of the measurements. If the subject was unable to perform very high or low flow rates, Silkoff method seemed to be the most feasible.

Roy et al. [21] have also compared different mathematical methods and found that C_ANO results are significantly model-dependent and median error between calculated and observed data is considerably lower in non-linear methods than mixed methods. Eckel et al. compared nine different mathematical methods in parameter estimation [41]. They suggested using non-linear least squares model with natural log transformation on both sides in parameter estimation. This can be considered as Silkoff method with natural log transformation on both sides. In their study, Pietropaoli method yielded the least number of negative C_ANO estimates which is in line with our study where Pietropaoli method yielded least outliers.

On average, the difference between measured and calculated F_ENO at 100 ml/s was very low. The difference would probably be higher at a lower flow of e.g. 50 ml/s where the relation between V_{NO} and V_E is more nonlinear and the absolute measured ppb values are higher. However, as the linear models do not apply at such low flow rates we decided to use the flow rate of 100 ml/s in assessing methods' degree of agreement with measured data.

4.3. Differences in NO parameters between the methods

There were some statistically significant differences in the NO parameters between the methods but the differences were usually quite small. This is in line with previous reports [21,41]. As there is no gold standard to assess the NO parameters it is difficult to say which of the methods yields best estimates.

The linear methods were quite well in line with each other as were the non-linear methods. The linear methods gave higher estimates for C_ANO and lower estimates for J_{aw}NO as compared with non-linear methods, but the Condorelli method gave smaller estimates for C_ANO and higher for J_{aw}NO. This is likely explained by the fact that the linear methods neglect the axial back-diffusion of NO from the conducting region into the alveolar region and our findings support this statement. The fact that the linear approximation of the governing equation of the two-compartment model is only an approximation and the relation between V_{NO} and V_E in T&G method and the relation between F_ENO and $1/V_E$ in Pietropaoli method is not perfectly linear, may also explain our results as this causes a minor overestimation of C_ANO and underestimation of J_{aw}NO [29]. In all subject groups Silkoff method gave similar results with the Condorelli method for C_ANO. Silkoff method yielded lower estimates for C_{aw}NO and higher estimates for D_{aw}NO as compared to H&M. This probably reflects the difference in the mathematical procedure how these parameters are solved in each of the methods.

4.4. Differences in NO parameters obtained using different flow rates

We found differences in NO parameters obtained using different flow rates. This is in line with previous results that NO parameters vary with different used flow rates [31,32]. We found that the choice of flow rates had more impact on the results of the linear T&G method than of the non-linear H&M.

4.5. Strengths and limitations

Our study has several strengths. We had a relatively large sample size, which provided us with statistical power. All measurements were performed by using the same NO analyzer within same research center, which possibly reduced variation. We had also four distinct subject groups, establishing the comparison of the feasibility of the methods in adults and children and healthy and diseased groups.

However, our study also has weaknesses. Our study included only young children and older adults, lacking young adults. According to previous studies, the flow independent NO parameters vary with age and this may have affected our results [42]. Roy et al. [21]suggested standardizing the number of used flow rates as it significantly affects the NO parameter values. We decided not to standardize our used flow rates as our objective was more to determine whether the parameters can be calculated at all by accommodating the plots if measurement errors were encountered. However, this finding must be taken account as a possible source of error. Another limitation of our study is that we did not have a real-time validity check for the fit between measured NO data and the mathematical methods but only a check that repeatable F_ENO values are obtained at each flow rate. A real-time check of the agreement with mathematical models would probably improve the feasibility of H&M method especially as it uses only three flows (two flows at a time for each step of the mathematical process) and is vulnerable to measurement errors even at one flow.

4.6. Conclusion

In conclusion, if the interest lies in differentiating central and peripheral NO sources and assessing $J_{aw}NO$ and C_ANO, based on the current results we recommend using the linear methods, as low flow rates can be avoided and the feasibility of the methods is good. The difference between Tsoukias & George and Pietropaoli methods does not seem relevant. However, Condorelli method with correction for axial back-diffusion of NO differed from other linear methods and may not be applicable in subjects with obstruction.

If $C_{aw}NO$ and $D_{aw}NO$ are of interest, we recommend using H&M method as only three flow rates are needed. However, due to its vulnerability, we recommend assessing the agreement between the model and measured data in real-time to ensure the quality of measurements, as the extreme flow rates are observed to be problematic to some subjects. If the subject has difficulties with the extremely low or high flow rates, we then recommend using the Silkoff method to improve feasibility. However, more flow rates and F_ENO measurements are then needed and the agreement between the model and the data may be poorer.

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We declare no conflicts of interest.

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7. Supplementary tables

Supplementary Table 1. Comparison of the methods' degree of agreement with measured data.

	Absolute values (ppb) of the difference between measured and calculated F_ENO-values at 100 ml/s					**p-values**					
	T&G	*Pietropaoli*	*Condorelli*	*Silkoff*	*H&M*	*Silkoff-H&M*	*Silkoff-T&G*	*Silkoff-Pietropaoli*	*H&M-T&G*	*H&M-Pietropaoli*	*T&G-Pietropaoli*
						T&G-Condorelli	*Pietropaoli-Condorelli*	*Silkoff-Condorelli*	*H&M-Condorelli*		
Healthy adults											
Median	0,42	0,08	0,17	0,49	0,03	0,000	1,000	0,000	0,000	0,164	0,000
IQR 1. - 3.	0,14 - 0,58	0,03 - 0,13	0,07 - 0,28	0,26 - 1,04	0,01 - 0,05	0,063	0,133	0,000	0,000		
Min	0,00	0,00	0,00	0,02	0,00						
Max	2,02	1,39	1,21	3,52	0,75						
Healthy children											
Median	0,13	0,03	0,08	0,28	0,01	0,000	1,000	0,000	0,000	0,041	0,000
IQR 1. - 3.	0,07 - 0,23	0,02 - 0,06	0,04 - 0,15	0,09 - 0,46	0,00 - 0,02	0,278	0,022	0,136	0,000		
Min	0,00	0,00	0,00	0,01	0,00						
Max	0,50	0,16	0,37	1,06	0,09						
Asbestos exposure											
Median	0,24	0,06	0,17	0,46	0,02	0,000	0,941	0,000	0,000	0,243	0,000
IQR 1. - 3.	0,10 - 0,41	0,02 - 0,09	0,07 - 0,28	0,20 - 0,84	0,01 - 0,04	0,735	0,000	0,005	0,000		
Min	0,02	0,00	0,00	0,02	0,00						
Max	0,96	0,20	0,63	5,88	0,16						
COPD											
Median	0,18	0,03	0,44	0,37	0,00	0,000	0,219	0,000	0,044	1,000	0,336
IQR 1. - 3.	0,08 - 0,37	0,01 - 0,08	0,17 - 0,84	0,17 - 0,82	0,01 - 0,02	0,162	0,000	1,000	0,000		
Min	0,00	0,00	0,01	0,03	0,00						
Max	2,25	0,44	6,81	8,36	0,06						

Supplementary Table 2. Comparison of central and peripheral nitric oxide parameters obtained by using four different mathematical methods in healthy adults.

Healthy Adults	**Parameter values**					**p-values**					
	T&G	Pietropaoli	Condorelli	Silkoff	H&M	Silkoff-H&M	Silkoff-T&G	Silkoff-Pietropaoli	H&M-T&G	H&M-Pietropaoli	T&G-Pietropaoli
						T&G-Condorelli	Pietropaoli - Condorelli	Silkoff-Condorelli	H&M-Condorelli		
D_{aw}NO (pl/s/ppb)						0,000					
Median				8,31	7,27						
IQR 1. - 3.				5,41 - 12,29	4,01 - 9,03						
Min				0,98	0,51						
Max				28,85	29,44						
C_{aw}NO (ppb)						0,000					
Median				119,10	152,15						
IQR 1. - 3.				88,12 - 177,36	107,26 - 296,20						
Min				26,95	36,04						
Max				535,91	859,18						
C_ANO (ppb)						0,077	0,000	0,000	0,000	0,001	0,196
Median	3,02	2,74	2,18	1,82	2,38	0,000	0,000	1,000	0,830		
IQR 1. - 3.	2,46 - 3,88	2,27 - 3,37	1,64 - 3,11	1,11 - 2,53	2,00 - 2,98						
Min	0,98	1,03	0,17	0,06	0,65						
Max	6,16	6,45	5,43	4,77	4,38						
J_{aw}NO (pl/s)						1,000	0,000	0,000	0,000	0,001	0,532
Median	806,67	859,08	1270,22	919,37	926,68	0,000	0,000	0,001	0,000		
IQR 1. - 3.	515,00 - 1010,00	583,28 - 1123,69	817,33 - 1602,67	764,97 - 1315,98	726,52 - 1312,12						
Min	155,56	150,00	248,89	240,63	272,85						
Max	3656,67	3517,74	3813,33	4262,83	3850,70						

Supplementary Table 3. Comparison of central and peripheral nitric oxide parameters obtained by using four different mathematical methods in healthy children.

Healthy children	**Parameter values**					**p-values**					
	T&G	Pietropaoli	Condorelli	Silkoff	H&M	Silkoff-H&M	Silkoff-T&G	Silkoff-Pietropaoli	H&M-T&G	H&M-Pietropaoli	T&G-Pietropaoli
						T&G-Condorelli	Pietropaoli-Condorelli	Silkoff-Condorelli	H&M-Condorelli		
$D_{aw}NO$ (pl/s/ppb)						0,000					
Median				8,56	6,49						
IQR 1. - 3.				4,75 - 14,42	3,48 - 11,67						
Min				0,28	0,86						
Max				35,47	27,14						
$C_{aw}NO$ (ppb)						0,000					
Median				53,60	57,28						
IQR 1. - 3.				34,53 - 103,43	40,83 - 131,78						
Min				15,01	17,91						
Max				596,29	727,88						
$C_{A}NO$ (ppb)						0,000	0,000	0,000	0,007	1,000	0,002
Median	2,18	2,16	1,85	1,70	2,13	0,000	0,000	1,000	0,000		
IQR 1. - 3.	1,90 - 2,89	1,84 - 2,66	1,56 - 2,53	1,30 - 2,28	1,79 - 2,50						
Min	0,90	0,96	0,80	0,58	0,41						
Max	4,45	4,74	3,53	3,59	4,36						
$J_{aw}NO$ (pl/s)						0,034	0,000	0,000	0,000	0,532	0,278
Median	336,67	378,46	533,33	445,66	449,84	0,000	0,000	0,014	0,000		
IQR 1. - 3.	246,67 - 580,00	275,77 - 602,31	394,67 - 928,00	326,98 - 682,70	301,48 - 651,88						
Min	80,00	71,54	128,00	164,52	122,38						
Max	1386,67	1375,38	1610,67	1227,00	1597,65						

Supplementary Table 4. Comparison of central and peripheral nitric oxide parameters obtained by using four different methods in subjects with a previous asbestos exposure.

Asbestos exposure	Parameter values					p-values					
	T&G	Pietropaoli	Condorelli	Silkoff	H&M	Silkoff-H&M	Silkoff-T&G	Silkoff-Pietropaoli	H&M-T&G	H&M-Pietropaoli	T&G-Pietropaoli
						T&G-Condorelli	Pietropaoli-Condorelli	Silkoff-Condorelli	H&M-Condorelli		
$D_{aw}NO$ (pl/s/ppb)						0,000					
Median				9,87	7,88						
IQR 1. - 3.				6,36 - 14,58	4,37 - 12,88						
Min				0,85	1,05						
Max				69,47	57,25						
$C_{aw}NO$ (ppb)						0,000					
Median				88,66	101,21						
IQR 1. - 3.				54,05 - 155,00	62,85 - 178,45						
Min				18,71	17,41						
Max				392,88	773,61						
$C_{A}NO$ (ppb)						0,130	0,000	0,000	0,000	0,032	0,047
Median	2,71	2,57	2,15	1,77	2,25	0,000	0,000	1,000	0,003		
IQR 1. - 3.	2,06 - 3,47	1,91 - 3,09	1,46 - 2,85	1,17 - 2,59	1,64 - 2,92						
Min	0,73	0,76	0,37	0,26	0,57						
Max	5,84	5,67	5,17	6,57	6,24						
$J_{aw}NO$ (pl/s)						1,000	0,000	0,000	0,000	0,000	0,209
Median	623,33	675,08	997,33	823,93	766,53	0,000	0,000	0,000	0,000		
IQR 1. - 3.	418,89 - 890,56	455,38 - 903,08	697,56 - 1464,00	601,31 - 1104,46	602,97 - 1085,62						
Min	186,67	229,74	298,67	283,53	250,63						
Max	1936,67	1955,90	3098,67	2729,35	2317,39						

Supplementary Table 5. Comparison of central and peripheral nitric oxide parameters obtained by using four different methods in subjects with COPD.

COPD	**Parameter values**					**p-values**					
	T&G	Pietropaoli	Condorelli	Silkoff	H&M	Silkoff-H&M	Silkoff-T&G	Silkoff-Pietropaoli	H&M-T&G	H&M-Pietropaoli	T&G-Pietropaoli
						T&G-Condorelli	Pietropaoli-Condorelli	Silkoff-Condorelli	H&M-Condorelli		
$D_{aw}NO$ (pl/s/ppb)						0,011					
Median				5,41	3,09						
IQR 1. - 3.				3,02 - 10,77	1,70 - 13,16						
Min				0,62	0,47						
Max				41,88	58,59						
$C_{aw}NO$ (ppb)						0,011					
Median				64,68	101,82						
IQR 1. - 3.				32,22 - 194,33	34,47 - 314,42						
Min				6,36	5,95						
Max				858,49	931,30						
$C_{A}NO$ (ppb)						1,000	0,000	0,003	0,000	0,006	1,000
Median	3,04	3,20	2,85	1,85	2,23	0,162	1,000	0,139	0,253		
IQR 1. - 3.	2,39 - 4,01	2,22 - 4,00	2,21 - 4,02	1,31 - 2,51	1,48 - 2,71						
Min	1,40	1,50	1,08	0,18	0,70						
Max	8,32	8,73	9,53	7,83	6,46						
$J_{aw}NO$ (pl/s)						0,442	0,000	0,002	0,062	0,831	1,000
Median	333,33	368,46	396,67	386,21	400,42	0,000	0,000	1,000	0,139		
IQR 1. - 3.	145,56 - 416,11	266,85 - 480,92	181,50 - 656,00	222,54 - 612,14	224,03 - 629,95						
Min	-100,00	46,15	-170,00	76,83	81,98						
Max	2113,33	2220,72	3381,33	4218,63	2462,62						