Repeatability and variation of the flow independent nitric oxide parameters

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Abstract

Introduction: Fractional exhaled nitric oxide ($F_E NO$) is a non-invasive marker of airway inflammation. Measuring $F_E NO$ at several flow rates enables the calculation of flow independent NO-parameters (alveolar NO concentration ($C_A NO$), bronchial flux of NO ($J_{aw} NO$), bronchial mucosal NO concentration ($C_{aw} NO$) and bronchial wall NO diffusion capacity ($D_{aw} NO$)) that are capable of partitioning the source and release mechanism of NO from the lower respiratory tract. However, the current literature on repeatability and normal variation of the NO-parameters is deficient, and this information is needed to develop the method towards clinical use.

Methods: We calculated NO-parameters in altogether 28 healthy subjects using two different mathematical methods and used three different study protocols to investigate: (i) repeatability of two consecutive measurements of NO parameters, (ii) within-day variation of the NO-parameters over one working day and (iii) day-to-day variation of the NO-parameters between consecutive days during course of a working week.

Results: $J_{aw} NO$ was the most repeatable among the NO-parameters, whereas $D_{aw} NO$ and $C_{aw} NO$ were notably least repeatable. $C_A NO$ was higher during the second consecutive measurement (1.22 vs 1.57 ppb, p=0.017). Both investigated mathematical methods yielded equally repeatable results. $J_{aw} NO$ was slightly higher in the afternoon compared to morning (716 vs 881 pl/s, p=0.01), but other parameters showed no diurnal variation. Upper 95 % limit for the day-to-day difference in the parameters in healthy subjects was about 1.2 ppb in $C_A NO$, 400 pl/s in $J_{aw} NO$, 92 ppb in $C_{aw} NO$ and 16 pl/s/ppb in $D_{aw} NO$.

Conclusions: This is the first study assessing short-time repeatability of the NO-parameters. Repeatability of the NO-parameters was good and day-to-day variation in NO-parameters was quite low. We recommend scheduling $F_E NO$-measurements at the same time of day, if possible, and in clinical use variation in NO-parameters above the normal limits found is this study suggest changes in diseases activity.

Introduction

Measuring fraction of exhaled nitric oxide (F_ENO) is widely studied and standardized [1,2] with official guidelines on its clinical use in diagnosis, monitoring and management of asthma [3,4]. Usually, F_ENO is measured at flow rate of 50 ml/s (F_ENO$_{50}$). However, F_ENO measured at a single flow rate does not tell the source of exhaled NO within the lower respiratory tract. Tsoukias & George introduced a two-compartment model of NO exchange dynamics of the lungs which is capable of partitioning the source and release mechanism of NO from the lower respiratory tract [5] if F_ENO is measured at multiple flow rates (extended F_ENO-measurement, eF_ENO-measurement). They also introduced a robust method of estimating alveolar NO concentration (C_ANO, ppb) and bronchial NO flux (J_{aw}NO, pl/s) by calculating slope and intercept, respectively, of a linear regression line between NO output and exhalation flow rate (T&G-method) [6]. This method is a linear approximation of the two-compartment model's non-linear equation and applies only with higher flow rates. Only methods using the non-linear equation of the two-compartment model allow calculation of bronchial mucosal NO concentration (C_{aw}NO, ppb) and airway diffusing capacity of NO (D_{aw}NO, pl/s/ppb) and hence the release mechanism of NO from the lower respiratory tract [7]. Högman and Meriläinen introduced a simple iteration method based on three flow rates to allow calculation of C_{aw}NO and D_{aw}NO [8]. These two methods have been recommended by the latest technical standard on F_ENO [1].

Extended F_ENO-measurement has been investigated for two decades, but there is still no clear consensus on all the technical aspects. NO-parameters calculated based on eF_ENO-measurement are known to vary depending on mathematical method and flow rates used [9,10], making the comparison of different research groups' results challenging. In addition, repeatability and normal variation of the NO-parameters over time are still to be better assessed. The terms repeatability, reproducibility and variation are not always used coherently in literature. Repeatability refers to agreement between results of two successive measurements carried under the same conditions of measurement while variation refers to difference in two measurements where the cause of difference is change in the measured object rather than error in measurement technique.

To our knowledge, the current literature on eF_ENO lacks studies assessing repeatability. There are some publications on within-day variation [11-13] but sample sizes are relatively small and results from both healthy and diseased subjects are combined in some studies. Day-to-day variation has been investigated between two consecutive days or two days with a longer interval [10-12,14-17], but there are no studies measuring NO-parameters on daily basis for several days consecutively. Knowledge on the repeatability of the measuring method and normal variation of NO parameters is of great importance for the development of eF_ENO measurement towards clinical applications, as it helps judging whether certain degree of change in NO parameters of a patient reflect changes in disease state or merely normal variation.

The purpose of this study was to determine the repeatability of eF_ENO-measurement and normal variation over time of the NO-parameters. This study had three different study protocols to investigate: (i) repeatability of two consecutive eF_ENO-measurements, (ii) within-day variation of the NO-parameters over one working day and (iii) day-to-day variation of the NO-parameters between consecutive days in course of a working week.

Methods

Subjects

All subjects were adults (over 18 years), non-smoking and healthy with regard to respiratory diseases and free of respiratory tract infections for at least 4 weeks. Subjects were asked not to eat, drink caffeinated beverages or heavily exercise within one hour prior the measurements. All subjects gave their written informed consent and the study was approved by the ethics committee of Tampere University Hospital (R17076).

Extended F_ENO-measurement

F_ENO was measured using an ozone-chemiluminescence analyzer (NOA 280, Sievers Instruments, Boulder, CO, USA). The analyzer was calibrated daily by using calibration gas with known NO concentration of 20.5 ppm (AGA, Lidingö, Sweden). Zero-calibration was run daily using filtered NO-free air. Both calibrations

were repeated twice successively. Computer controlled flow restrictor was calibrated before every subject at flow rates of 100 and 300 ml/s by using a 3 ± 0.03 -liter syringe. Subjects were asked to flush their mouth and pharynx with water prior the measurements. Subjects were in a sitting position and were instructed to inhale through mouth to total lung capacity and then exhale through the mouthpiece steadily. Mouthpiece with a bacterial filter was connected to a computer-controlled flow restrictor [18]. The device measures flow rate in real-time and adjusts flow resistance to either increase or decrease the flow towards the target level to adjust for changes in the subjects' exhalation effort as long as the mouth pressure remained between 5 and 20 cmH$_2$O [19]. Subjects received visual feedback on mouth pressure on a computer screen and were asked to keep the pressure steady between 5 and 20 cmH$_2$O. Researcher followed the F$_E$NO vs time -curve in real time and instructed the subject to maintain the mouth pressure steady until the curve had reached a steady plateau or the subject was run out of breath. NO concentration was then read from the plateau and the same flow rate was repeated. If a steady plateau was not reached, the subject was allowed to attempt the flow rate third time. If still no steady plateau was formed, the flow rate was discarded from the analysis. F$_E$NO-measurement was performed at eight different flow rates: 10, 20, 30, 50, 100, 200, 300 and 400 ml/s. F$_E$NO was measured 2-3 times at every flow rate and mean F$_E$NO at each flow rate was calculated and used as the result. Ambient air NO-concentration was measured prior every subject.

The method of Tsoukias & George (T&G) was used on F$_E$NO values at flow rates 100-400 ml/s to calculate J$_{aw}$NO and C$_A$NO. The linearity of NO-output vs flow rate at flows ≥ 100 ml/s was evaluated and correlation factor $r < 0.9$ was used as an exclusion criterion. Högman & Meriläinen -algorithm (HMA) based on flow rates of 10, 100 and 400 ml/s and was used to calculate J$_{aw}$NO, C$_A$NO, C$_{aw}$NO and D$_{aw}$NO [20]. HMA has a built-in quality control and as a further quality control, C$_A$NO-values below zero and C$_{aw}$NO > 1000 ppb were excluded.

Study protocols and analyses

Short-term repeatability of two consecutive eF$_E$NO-measurements

Two eF$_E$NO-measurements were performed with only a short few minute break between the measurements if needed. NO-parameters were calculated using both HMA and T&G methods. Repeatability of each NO-parameter was assessed by three ways: calculating coefficient of repeatability (CR), calculating within-subject coefficient of variation (CV) and by assessing upper and lower 95% limits of agreement between the first and second measurement by Bland-Altman plots. CR describes the confidence interval where the absolute difference between two repeated measurements is expected to lie with 95% probability and was calculated as

$CR = 1.96\sqrt{\frac{\sum(d2-d1)^2}{n}}$, where d1 is the first and d2 the second measurement and n is the number of subjects

[21]. CV was calculated as CV = within-subject SD / within-subject mean, where SD is standard deviation. We also analyzed differences between group means or medians of the NO parameters from two consecutive eF$_E$NO-measurements by using paired t-test or Wilcoxon's signed-rank test. To compare the repeatability of the two used methods (T&G and HMA), the CV-values for these methods were compared by paired t-test or Wilcoxon's signed-rank test.

Within-day variation of the NO-parameters during working day

Within-day variation of the NO-parameters during a working day was investigated by measuring eF$_E$NO three times in course of one working day. Measurements were performed between 7:00-10:00, 10:00-13:00 and 13:00-16:00. There was at least a two-hour gap between measurements in each subject. The variation of the NO-parameters during the day was evaluated by calculating CV. The differences in parameter means or medians between different times of day were assessed with a repeated measures one-way ANOVA (Greenhouse-Geisser correction was used if the data violated the assumption of sphericity) or non-parametric Friedmans's 2-way ANOVA by ranks.

Day-to-day variation of the NO-parameters between consecutive days in course of one week

eF$_E$NO was measured on 3-5 separate days during one working week and most of the subjects were able to participate on 4 consecutive days. The measurements were performed at the same time daily for each subject

to overcome possible effects of diurnal variation. The differences in parameter means or medians between different consecutive days were assessed using a generalized linear mixed model, with the measurement session set as a fixed effect factor. Some subjects had only 3 measurements and repeated ANOVA or Friedman's test would have excluded subjects with missing data entirely from the analyses. To compare the day-to-day variation of the two methods used (T&G and HMA), the mean CV-values obtained by these methods were compared by paired T-test or Wilcoxon's signed-rank test.

Day-to-day variation was also assessed by calculating variation coefficient: $v = 1.96\sqrt{2} * Sv$, where Sv is variation standard deviation. Sv was calculated as: $Sv = \sqrt{\sum_{i=1}^{p} s_i^2 / p}$, where S_i is within-subject standard deviation and p is number of subjects.

Sample sizes and tests for normality
Required sample sizes for the study protocols were calculated based on an allowance of 5 % α-error and 90 % statistical power in detecting differences between measurements that are 0.25 times the population's standard deviation. Based on this, sample sizes of 22, 11 and 8 subjects were aimed for in testing repeatability, with-in-day and day-to-day variation, respectively. Shapiro-Wilk's test was deployed to test for the normality of the parameters. All statistical analysis was calculated using IBM SPSS. NO-parameters were calculated using Microsoft Excel. Bland-Altman -plots were drawn with R package "blandr" [22].

Results
Altogether, 28 subjects participated in this study and most of them participated in more than one protocol. Subjects were mostly young adults (subject characteristics in Table 1). Ambient air NO-concentration was on average 0.47 ± 0.56 ppb and free of within-day variation and 2.5 ppb at highest during the measurements included in analyses. On one occasion ambient air NO-concentration was 114 ppb but results obtained on that day were excluded from the analyses.

Table 1. Subject characteristics.

| | **Protocol** | | |
Variables	*Repeatability between two consecutive measurements*	*Within-day variation*	*Day-to-day variation*
n (m/f)	25 (16/9)	14 (9/5)	13 (8/5)
Age (years)*	24 20–64	23 21–50	24 20–34
Height (cm)	174 ± 9.98	175 ± 10.6	177 ± 8.9
Weight (kg)	71.0 ± 9.50	72 ± 10.5	70.9 ± 10.1

*Age presented as median min–max, other variables as mean ± SD

Repeatability between two consecutive eF$_E$NO-measurements
J$_{aw}$NO and single F$_E$NO-values were most repeatable regarding the within-subject CV-values (Table 2). CV of J$_{aw}$NO was smallest among the parameters calculated with both methods (HMA and T&G). CV of D$_{aw}$NO and C$_{aw}$NO were notably higher than CV of the other parameters. There was no statistically significant difference in repeatability of the NO parameters between the two mathematical methods (HMA and T&G) based on within-subject CVs obtained (J$_{aw}$NO p = 0.741, C$_A$NO p = 0.058). Also, CR related to measured means was distinctly smaller in J$_{aw}$NO compared to the other parameters.

C$_A$NO was on average higher at the second measurement (T&G p = 0.017, HMA p = 0.028). J$_{aw}$NO showed also an increasing trend between the two measurements, but the difference was not statistically significant. F$_E$NO$_{10}$ and F$_E$NO$_{400}$ were also statistically significantly higher during the second measurement (p = 0.001, p

< 0.001). Figures 1 and 2 present the Bland-Altman plots for the repeatability of the parameters and eFigure 1 in e-supplement present these for single F_ENO-values.

Table 2. Repeatability of the NO-parameters between two consecutive eF_ENO-measurements.

Parameter	n	1. measurement	2. measurement	p-value	Within-subject CV[#]	Limits of agreement*	CR[¤]
$J_{aw}NO_{T\&G\ (pl/s)}$	25	907 ± 418	922 ± 418	0.218	4.5% ± 4.7%	-154–119	142
$C_ANO_{T\&G\ (ppb)}$	25	1.22 (0.98–2.00)	1.57 (1.19–1.92)	0.017†	9.6% ± 7.7%	-0.76–0.41	0.729
$J_{aw}NO_{HMA\ (pl/s)}$	21	850 ± 397	864 ± 383	0.526	4.5% ± 2.9%	-173–144	157
$C_ANO_{HMA\ (ppb)}$	21	1.14 (0.83–1.73)	1.26 (0.97–1.95)	0.028†	13.7% ± 14.3%	-1.15–0.71	1.00
$C_{aw}NO_{HMA\ (ppb)}$	21	115 (79.9–124)	138 (97.2–215)	0.073	14.9% ± 12.2%	-259–178	227
$D_{aw}NO_{HMA\ (pl/s/ppb)}$	21	7.28 ± 3.46	6.80 ± 4.04	0.280	17.0% ± 14.5%	-4.92–5.88	5.36
$F_ENO_{10\ (ppb)}$	23	66.6 ± 31.5	70.5 ± 33.4	0.001†	3.6% ± 2.4%	-13.0–5.21	11.7
$F_ENO_{50\ (ppb)}$	25	19.4 ± 8.58	19.8 ± 8.27	0.175	3.4% ± 3.0%	-3.32–2.49	2.96
$F_ENO_{400\ (ppb)}$	25	3.83 ± 1.46	4.03 ± 0.339	<0.001†	3.9% ± 3.3%	-0.66–0.27	0.601

[#] CV Coefficient of variation, [¤] CR Coefficient of repeatability, * Limits of agreement from the Bland-Altman -analysis, † p < 0.05, results reported as mean ± SD or median (1.st–3.rd interquartile range)
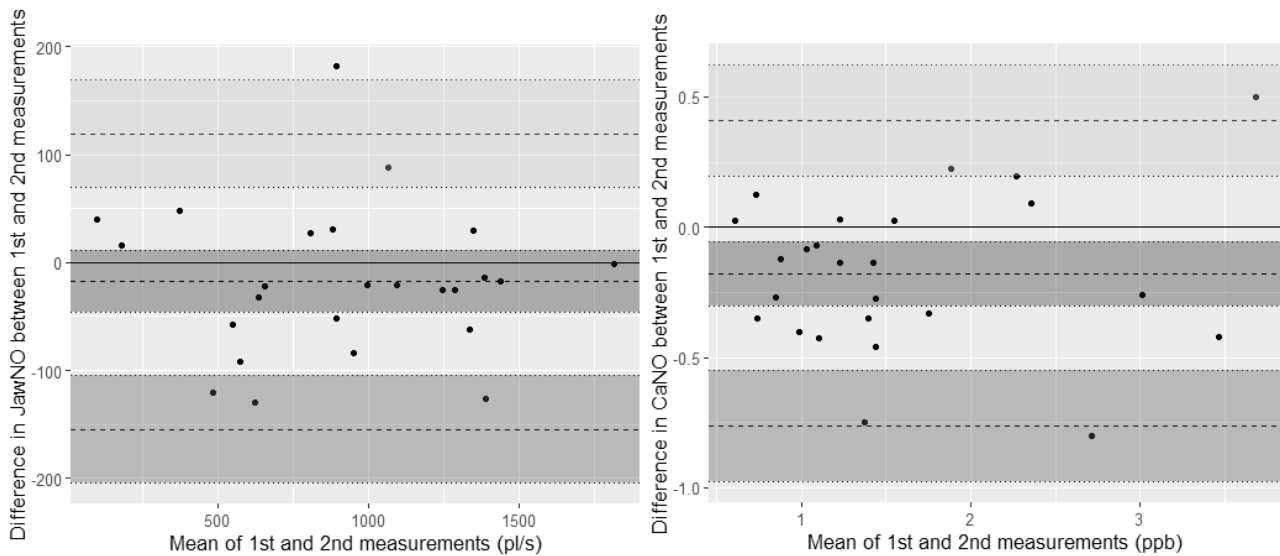

Figure 1. Bland-Altman plots of C_ANO and J_{aw}NO from two consecutive measurements using T&G-method.
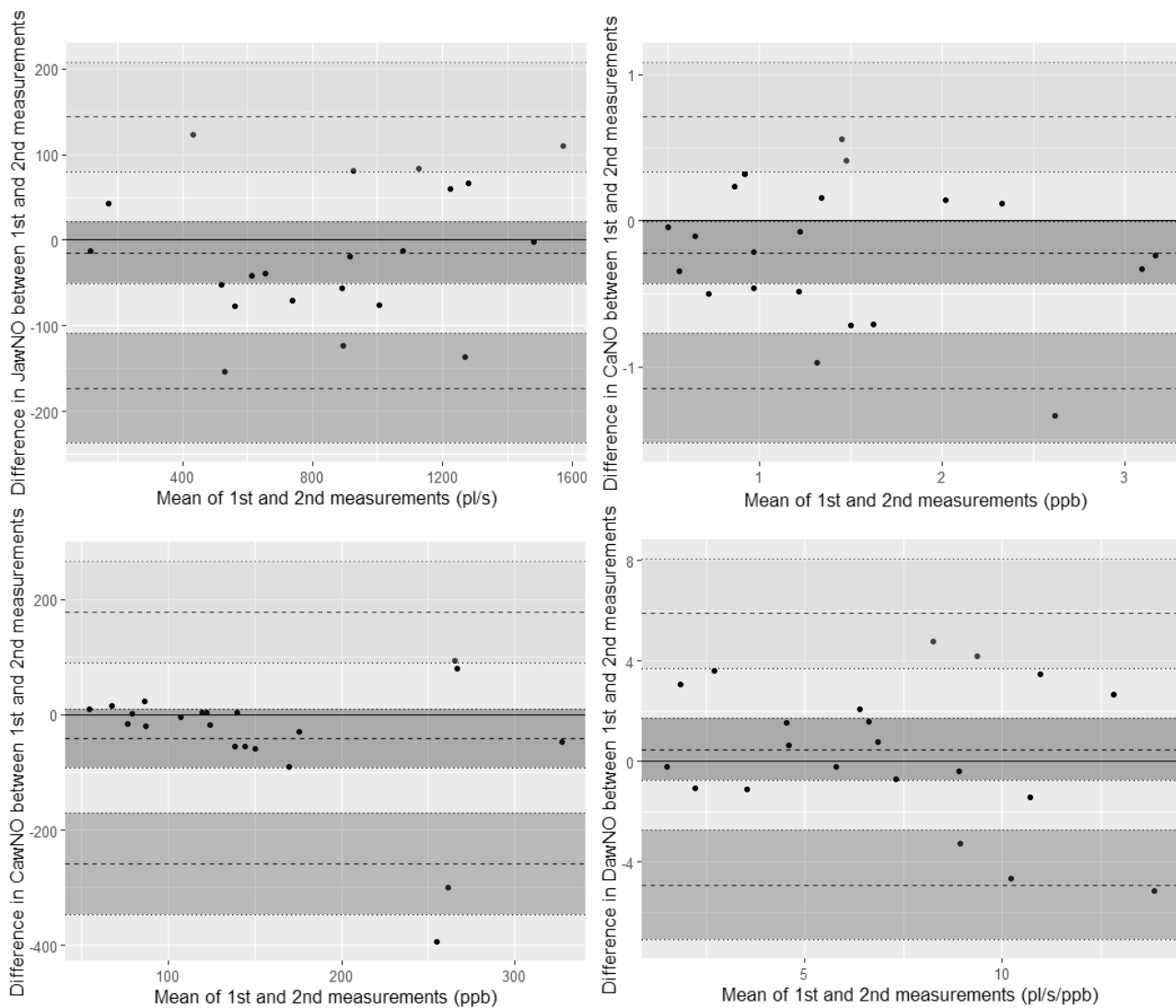
Figure 2. Bland-Altman plots of $J_{aw}NO$, C_ANO, $C_{aw}NO$ and $D_{aw}NO$ from two consecutive measurements using HMA-method.

Within-day-variation of the NO-parameters over a working day

A small but statistically significant increase was observed in $J_{aw}NO$ during a working day. $J_{aw}NO$ was 166 pl/s higher in afternoon compared to morning (p = 0.024) with the T&G-method and 223 pl/s with the HMA-method (p = 0.023). There were no significant differences in other calculated NO-parameters between different times of day (Table 3 and Figures 3-4). F_ENO_{10} was also increased in the afternoon compared to morning (F_ENO-values presented in eFigure 2).

Table 3. Diurnal variation of the NO-parameters over a working day.

Parameter	n	Morning (7:00 to 10:00)	Noon (10:00 to 13:00)	Afternoon (13:00 to 16:00)	p-value[¤]	Within subject CV
$J_{aw}NO_{T\&G\ (pl/s)}$	12	716 ± 387	819 ± 458	881 ± 425	0.01†	13.7% ± 6.7%
$C_ANO_{T\&G\ (ppb)}$	12	1.90 ± 0.81	1.68 ± 0.70	1.90 ± 0.66	0.387	16.0% ± 10.8%
$J_{aw}NO_{HMA\ (pl/s)}$	12	781 ± 344	939 ± 425	1005 ± 389	0.005†	14.3% ± 7.3%
$C_ANO_{HMA\ (ppb)}$	12	1.62 ± 0.86	1.15 ± 0.54	1.40 ± 0.72	0.236	35.8% ± 17.7%
$C_{aw}NO_{HMA\ (ppb)}$	12	135 94.1 183	143 73.5/208	126 85.9/299	0.920	29.4% ± 20.5%
$D_{aw}NO_{HMA\ (pl/s/ppb)}$	12	5.20 3.20/8.40	5.93 3.53/7.78	5.90 4.65/12.32	0.205	30.3% ± 14.6%
$F_ENO_{10\ (ppb)}$	14	59.0 ± 27.6	65.9 ± 34.3	69.0 ± 32.8	0.038†	10.2% ± 5.8%
$F_ENO_{50\ (ppb)}$	14	16.7 ± 7.9	18.5 ± 9.3	19.7 ± 8.7	0.051	9.0% ± 7.4%
$F_ENO_{400\ (ppb)}$	14	3.49 ± 1.33	3.49 ± 1.38	3.71 ± 1.23	0.366	9.8% ± 4.6%

¤ overall p-value, † $p<0.05$, results reported as mean ± SD or median 1.st/3.rd interquartile range
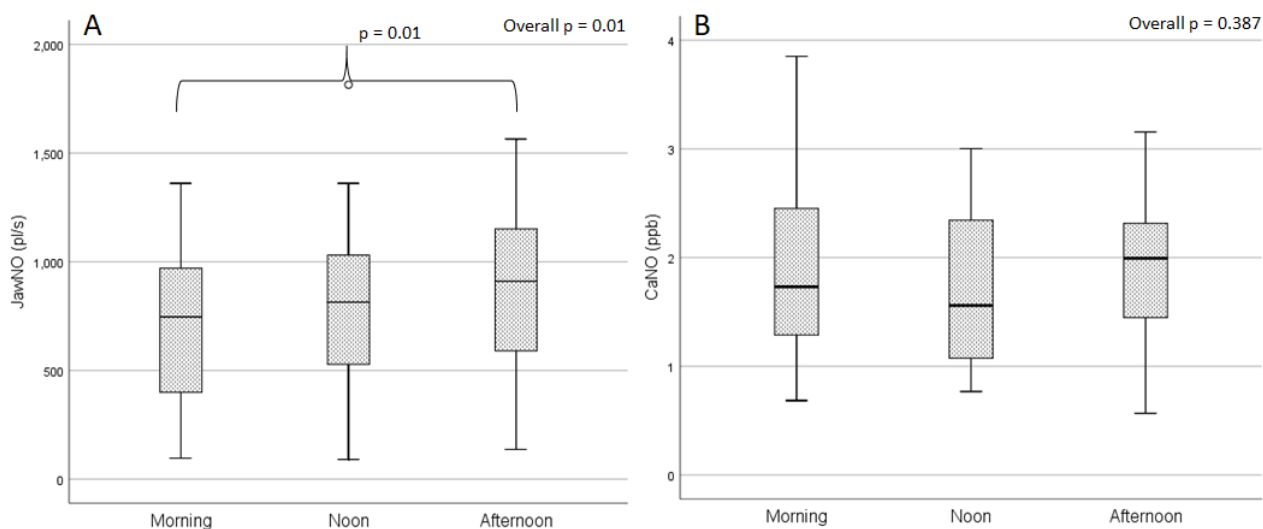

Figure 3. Diurnal variation of the NO-parameters calculated with T&G-method.
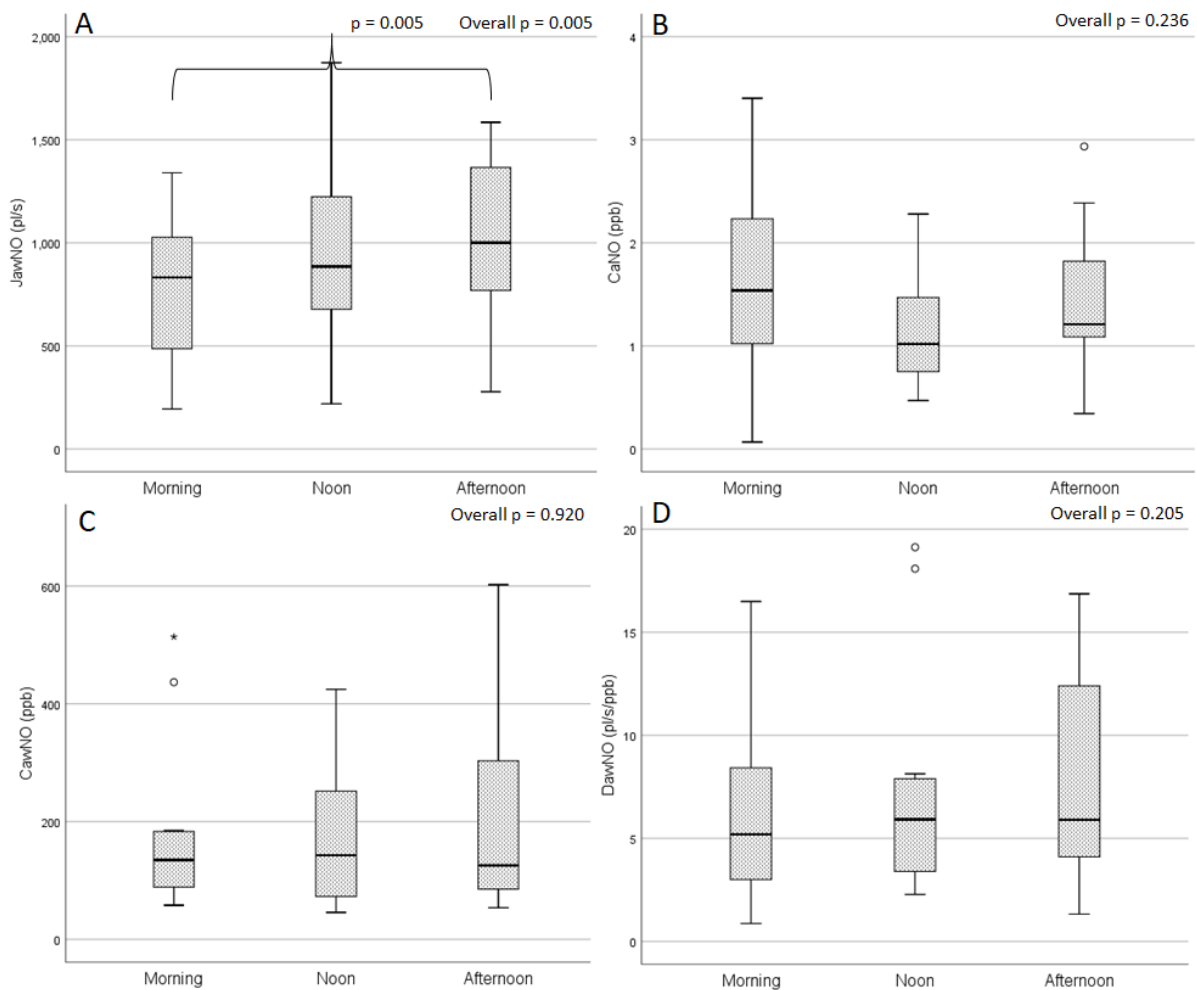
Figure 4. Diurnal variation of the NO-parameters calculated with HMA-method.

Day-to-day variation of the NO-parameters between consecutive days in course of one working week

No statistically significant difference was observed in any calculated NO-parameters between the measurements at consecutive days (Table 4, e-Figure 3-5). Naturally, the within-subject CV-values seemed clearly higher compared to the short-time study protocols, especially when HMA was used to calculate the parameters. HMA yielded significantly higher within-subject CV-values for C_ANO than T&G (p = 0.001) but the within-subject CV-values of $J_{aw}NO$ showed no difference (p=0.285). Variation coefficient of $J_{aw}NO$ was clearly smallest when compared to its mean. On the other hand, variation coefficients of $D_{aw}NO$ and $C_{aw}NO$ were even larger than their mean values. Variation coefficients seemed smaller when the parameters were calculated using the T&G-method (295 vs 397 pl/s, 1.17 vs 1.20 ppb).

Table 4. Day-to-day variation of the NO-parameters.

Variables	n	Within-subject CV	Mean of all days	Variation coefficient*
$J_{aw}NO_{T\&G}$	13	15.4% ± 8.4%	767 pl/s	295 pl/s
$C_ANO_{T\&G}$	13	19.2% ± 11.4%	1.6 ppb	1.17 ppb
$J_{aw}NO_{HMA}$	12	16.0% ± 9.3%	855 pl/s	397 pl/s
C_ANO_{HMA}	12	38.0% ± 21.4%	1.02 ppb	1.20 ppb
$C_{aw}NO_{HMA}$	12	31.1% ± 13.1%	86 ppb	92.1 ppb
$D_{aw}NO_{HMA}$	12	36.6% ± 19.0%	12.8 pl/s/ppb	15.7 pl/s/ppb
F_ENO_{10}	13	13.0% ± 6.8%	53.6 ppb	21.9 ppb
F_ENO_{50}	13	9.9% ± 4.0%	15.5 ppb	4.10 ppb
F_ENO_{400}	13	9.4% ± 5.9%	3.08 ppb	1.12 ppb

* variation coefficient: $v = \mathbf{1.96\sqrt{2}} * \boldsymbol{Sv}$, where $\boldsymbol{Sv} = \sqrt{\sum_{i=1}^{p} s_i^2/p}$ and where S_i is within-subject standard deviation and p is number of subjects. Results reported as mean ± SD or mean.

Discussion

To develop eF_ENO-measurement towards clinical applications, it is important to know the repeatability of the method and normal variation of the NO-parameters. In this study we found that repeatability of eF_ENO-measurement was good and day-to-day variation of NO-parameters in healthy subjects was quite low. The most repeatable NO-parameter was $J_{aw}NO$, whereas $D_{aw}NO$ and $C_{aw}NO$ were notably least repeatable and most variable. No significant difference between the two methods (HMA and T&G) was observed when comparing their repeatability. A small difference was observed between the morning and afternoon $J_{aw}NO$-values, but the other parameters showed no significant change during one working day. As might be expected, no differences between the consecutive days during working week were discovered.

Repeatability

C_ANO, F_ENO_{10} and F_ENO_{400} were on average higher at the latter consecutive eF_ENO-measurement, while there was no such difference in other NO-parameters. One explanation for the increase in C_ANO, F_ENO_{10} and F_ENO_{400} during the second measurement is that they all involve the most challenging flow rates for the subject: F_ENO_{10} requiring the longest steady blow and F_ENO_{400} usually requiring emptying one's lung capacity to the most. The problem with the flow rates that require emptying one's lung capacity to the most is that the F_ENO vs time curve does not always reach a steady plateau but instead may form a declining profile. Estimating plateau becomes more inaccurate and if the subject was fatigued during the latter measurement and performing shorter blows, estimated F_ENO-plateau could have been higher. Calculation of C_ANO is highly dependent on F_ENO at the highest flow rate used and the above mentioned phenomenon may have resulted in higher C_ANO during the second measurement.

Ascending or descending of the F_ENO-plateau as the subject fatigues depends on the shape of the F_ENO vs time curve. If the curve is declining in profile, the plateau of F_ENO remains at higher level as subject fatigues and performs shorter blows. We often noticed F_ENO vs time -curve to be declining in the higher flow rates. This is because as the subject begins to blow, higher NO levels from the oropharynx and upper airways create a peak in the beginning of the NO-curve. When subject exhales through the mouthpiece, air from less NO-rich peripheral part of the lung dilutes the NO-concentration, creating a declining curve finally reaching a steady plateau. However, this applies better for the higher flow rates when measured F_ENO is low and could explain increase in C_ANO as calculation of C_ANO is highly dependent on F_ENO measured at the higher flow rates.

$J_{aw}NO$ was most repeatable among the NO-parameters. This may be due to that $J_{aw}NO$ is calculated using the easily performed flow rates (100-400 ml/s) and it shows least variation when different combinations of flow rates are used [10] and thus one unsuccessful measurement point may not have had such significant effect on

$J_{aw}NO$. This could explain why a significant increase in $J_{aw}NO$ was not observed even if elevated C_ANO was caused by fatigued subjects. On the other hand, $D_{aw}NO$ and $C_{aw}NO$ were the least repeatable as calculation of these depend heavily on F_ENO measured at the low flow rates (10 ml/s in this study).

No statistically significant difference was observed in repeatability between the compared methods. However, CVs of C_ANO were almost statistically significantly higher with HMA compared to T&G (p = 0.058). We believe that the reason behind this lies in the number of used measurement points in the parameter calculation. HMA uses 3 flow rates whereas T&G uses 4 in this study. With less measurement points, inaccuracy in the parameter estimation increases, possibly explaining the higher CV values. Secondly, C_ANO was less repeatable compared to $J_{aw}NO$ with both estimation methods.

In this study, the subjects were mostly young, healthy and well cooperative. Nevertheless, the F_ENO-measurements were sometimes found challenging to perform, especially the extremely low and high flow rates. The low flow rates (10–20 ml/s) seemed to cause most difficulties. The low flow rates required sometimes long periods (up to 60 seconds) of slow and steady exhalation and subjects run out of breath before reaching a steady plateau. Sometimes subjects found it difficult to keep their tongue and pharynx steady during the low flow rates. Movement of tongue caused peak in the F_ENO vs time -curve and it hindered the formation of a steady plateau. Some subjects had also problems with the highest flow rate (400 ml/s). This was probably due to the lack of enough lung capacity for steady plateau to form. Subjects felt fatigued especially during the low flow rates of the second consecutive measurement and sometimes air tended to leak between lips and mouth piece. These problems emerge especially in a research protocol like this where two consecutive series of eF_ENO-measurements with 8 flows are done. In clinical use, one eF_ENO-measurement with less flows is surely easier for subjects to perform.

The latest technical standard on F_ENO measurement suggests using HMA and T&G methods for calculating NO parameters. These methods require flow rates of 10, 100 and 400 ml/s, and 100 – 400 ml/s, respectively. As this study was originally designed and initiated before the publication of the ERS technical standard, we had included also flow rates of 20 and 30 ml/s to the protocol. These were among the most challenging flow rates for subjects to perform and they unnecessarily exhausted the subjects and may have increased variation to the results.

To our knowledge, this is the first study investigating short time repeatability of the NO-parameters in healthy subjects. F_ENO_{50}-measurement has already been investigated and it is known to be highly repeatable [23], reproducible and free of within-day and day-to-day variation [23,24].

Within-day variation

We observed a small increase in $J_{aw}NO$ towards afternoon. We can only speculate the reason for increase in $J_{aw}NO$ during a working day in this study. $J_{aw}NO$ is determined by $C_{aw}NO$ and $D_{aw}NO$, of which both showed a mild but not significant increase during the day. Possible increase in $C_{aw}NO$ might be related to circadian rhythm of cortisol level (NO synthesis by iNOS is downregulated by glucocorticoids [25]) and the peak for cortisol secretion is about at the time of waking up [26]. Also, daytime exposure to different inhaled irritants might trigger inflammatory responses in airway epithelium and traffic related air pollutants are known to cause inflammation in peripheral airways and increased C_ANO-levels [27]. Other possible biological explanations could be related to subjects' diet. Up to 89 % increased F_ENO-levels are measured after nitrate rich meal and half of the subjects had increased F_ENO-levels even 3 hours after the meal [28]. Oral sodium nitrate intake has been noticed to increase NO-parameters $J_{aw}NO$ and C_ANO [29]. In this study, we instructed the subject to refrain from eating at least 1 hour prior the measurement. Chlorhexidine wash is noticed to lower $J_{aw}NO$ and normalize C_ANO after nitrate intake [29] but in this study, we used water for mouth wash prior the measurement. Day-time nitrate intake is one potential factor explaining our findings. Finnish breakfast usually consists of carbohydrate rich food (e.g. porridge, bread) low on nitrates. Nitrate intake during lunch may have increased the afternoon F_ENO and thus $J_{aw}NO$ levels. Changes in $D_{aw}NO$ are more complex since $D_{aw}NO$ is determined by both physical diffusivity of NO in airway mucosa and also the surface area of airway mucosa contributing to NO production [5]. Theoretically the same inhaled irritants that trigger inflammatory activity

to enhance NO production might increase $D_{aw}NO$ by activating iNOS also in more peripheral parts of airways and by increasing the surface area of NO production.

In addition to biological explanations, there is also theoretical possibility of calibration drift. However, we consider this quite unlikely. Ambient air NO-concentration was very low (0.47 ± 0.56 ppb) and was free of diurnal variation. Theoretically, ambient air NO could have decreased during the day, masking upward calibration drift. However, the NO-concentration in the morning was so low that possible decrease in ambient air NO during the day would be insufficient to mask such high calibration drift causing the observed increase in the NO-parameters.

To our knowledge, there are only a few studies investigating diurnal variation of the NO-parameters in healthy subjects. Brindicci et al. observed no significant diurnal variation in the NO-parameters, but the sample size was relatively small (n=9) [11]. In another study, asthmatics were mixed with healthy subjects but still no significant diurnal variation was observed [12]. On the other hand, Duong et al. noticed a significant increase in C_ANO and F_ENO_{50} between 8 p.m. and 6. a.m. of the day after [13]. Circadian variation of F_ENO_{50} has been investigated in more detail in healthy and asthmatic subjects and no statistically significant variation has been observed [24,30-32]. In these studies, F_ENO_{50} seems to have a decreasing trend during the day. Circadian variation in healthy subjects' sputum and blood eosinophils have also been studied but no significant variation has been observed [33].

According to our results, it would be recommended to schedule eF_ENO-measurements at the same time of day for each patient in future studies to overcome the possible bias caused by normal within-day variation. Scheduling of the eF_ENO-measurement becomes especially important if eF_ENO-measurement would be used to follow up treatment responses. However, the possible normal diurnal rhythm of the NO-parameters still needs to be further studied as the current literature has somewhat conflicting results.

Day-to-day variation

As expected, day-to-day variation seemed notably higher than the variation of two consecutive measurements. $J_{aw}NO$ among single F_ENO-values had least day-to-day variation compared to other parameters. $D_{aw}NO$ and $C_{aw}NO$ had relatively high variation coefficients, and thereby changes in these variables in subjects with pulmonary disease need to be quite large to be differentiated from normal variation. $J_{aw}NO$ and C_ANO on the other hand had lower variation coefficients, making these more usable for clinical use. CV for C_ANO seemed clearly higher with HMA compared to T&G. We believe that the amount of measurement points explains differences between these methods' CVs as discussed above. In clinical practice the method should be selected based on whether interest lies on JawNO and CaNO only (T&G is enough and may have less variation regarding CaNO) or if also CawNO and DawNO are needed (HMA should be used). No differences were observed in average values of the NO parameters between the consecutive days. To our knowledge, day-to-day variation of the NO-parameters has not been previously studied. However, F_ENO measurements have been noticed to have low variability when measured in 6 consecutive days in both healthy and asthmatic subjects [34].

Variation of the NO-parameters over varying time scales have been investigated in multiple studies. They all state that all the NO-parameters have little variation over time and it seems that $J_{aw}NO$ has least variation, whereas C_ANO and the other parameters show greater variation over time [11,12,15-17,35]. Some studies investigated only C_ANO and the variation was shown to be low [14,36]. Single F_ENO-values seemed less variable than the NO-parameters and this is possibly due to that the NO-parameters are calculated from multiple F_ENO-measurements and inaccuracy accumulates when multiple measurement points, each having their own random error, are used. This is especially an issue with $D_{aw}NO$ and $C_{aw}NO$ that are calculated based on an exponential function.

One potent source of error is that the subjects had different eating and exercise habits prior the measurements in consecutive days and this may have increased the observed inter-day variation. Subjects were instructed to avoid eating and heavily exercise at least one hour prior the eF_ENO-measurements. Physical exercise is known

to influence the NO-parameters. Shin et al. found $D_{aw}NO$ to increase, whereas $J_{aw}NO$ and C_ANO were decreased after high-intensity exercise [37]. $J_{aw}NO$ and C_ANO are also noticed to decrease in marathoners after a marathon run but on the other hand, these marathoners had higher baseline levels of C_ANO and $J_{aw}NO$ [38]. There are also studies reporting no change in F_ENO-levels after exercise [39] and the current literature overall is somewhat conflicting. However, exercise is reported to have both acute and long-term effects on the NO-parameters. Acute effects were tried to be minimized by avoiding exercise in one hour prior measurements. In clinical use, patients should be asked about their exercising habits

Some bias may arise from that the subjects learned to perform the F_ENO-measurement better during the study. This was especially true for the subjects that participated in more than one study protocol and this could be especially noticeable with the extreme flow rates. The possible learning effect may become a problem if the subject performs the eF_ENO-measurement multiple times and treatment is tailored according to changes in the results or disease progression is monitored with repetitive measurements. However, no difference in average values of the parameters was noticed between the consecutive days, which speaks against the possible learning effect. Kharitonov et al. investigated the learning effect in F_ENO-measurement but no significant effect was observed [24]**.** However, asthmatic subjects seemed to have a decreasing trend during the consecutive measurement situations.

Future research
Preferably our findings on repeatability and normal variation of NO parameters should be confirmed in another study. On top of only verifying the results in healthy subjects, further studies in subjects with different pulmonary diseases are needed to assess the relation between change in NO parameters and change in disease activity or disease progression.

Conclusion
In conclusion, short-term repeatability of eF_ENO-measurement was good and day-to-day variation in NO-parameters in healthy subjects was quite low. Of the NO-parameters, $J_{aw}NO$ was the most repeatable, whereas $D_{aw}NO$ and $C_{aw}NO$ were notably least repeatable. Both investigated methods (T&G and HMA) yielded equally repeatable results in short-term, but within-subject CV for C_ANO was higher with HMA than with T&G. A small difference between morning and afternoon values of $J_{aw}NO$ and F_ENO_{50} was observed and if possible, we recommend scheduling repeated F_ENO-measurements at the same time of day. The upper 95 % limit for normal day-to-day variation in NO parameters was about 1.2 ppb in C_ANO, 400 pl/s in $J_{aw}NO$, 92 ppb in $C_{aw}NO$ or 16 pl/s/ppb in $D_{aw}NO$. In clinical use a between-visits change in NO parameters above these limits suggest changes in diseases activity.

Conflicts of interest
We declare no conflicts of interest.

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