

# Flow-independent nitric oxide parameters in asthma: a systematic review and meta-analysis

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## Abstract

## Introduction

Fractional exhaled nitric oxide ( $F_{E}NO$ ) has been proposed as a non-invasive marker of inflammation in the lungs. Measuring  $F_{E}NO$  at several flow rates enables the calculation of flow independent NO-parameters that describe the NO-exchange dynamics of the lungs more precisely. The purpose of this study was to compare the NO-parameters between asthmatics and healthy subjects in a systematic review and meta-analysis.

## Methods

A systematic search was performed in Ovid Medline, Web of Science, Scopus and Cochrane Library databases. All studies with asthmatic and healthy control groups with at least one NO-parameter calculated were included.

## Results

From 1137 identified studies, 33 were included in the meta-analysis. All 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 ( $D_{aw}NO$ )) were found increased in glucocorticoid-treated and glucocorticoid-naïve asthma.  $J_{aw}NO$  and  $C_{A}NO$  were most notably increased in both study groups. Elevation of  $D_{aw}NO$  and  $C_{aw}NO$  seemed less prominent in both asthma groups.

## Discussion

We found that all the NO-parameters are elevated in asthma as compared to healthy subjects. However, results were highly heterogenous and the evidence on  $C_{aw}NO$  and  $D_{aw}NO$  is still quite feeble due to only few studies reporting them. To gain more knowledge on the NO-parameters in asthma, non-linear methods and standardized study protocols should be used in future studies.

# Introduction

Nitric oxide (NO) was first discovered in the exhaled breath of humans in 1991 (Gustafsson et al 1991) and two years later fractional exhaled NO ( $F_{E}NO$ ) levels were found to be increased in subjects with asthma (Alving et al 1993). Ever since,  $F_{E}NO$ -measurement has been vigorously studied as a possible marker of pulmonary inflammation. Today,  $F_{E}NO$  measurement is well standardized and in clinical use in diagnostics and management of asthma and there are systematic reviews and official guidelines on this topic (GINA Report 2018, National Institute for Health and Clinical Excellence 2017). The drawback of  $F_{E}NO$  measurement at a single flow rate is its inability to detect the source and release mechanism of NO from the lower respiratory tract.

Tsoukias and George developed the two-compartment model of pulmonary NO-dynamics in 1998 (Tsoukias and George 1998). In this model, the lungs are divided into two different regions or compartments: expansible alveolar region representing the alveoli and respiratory bronchioles (generation 18 and beyond in Weibel's lung model (Weibel E. 1963)) and rigid airway region representing the conducting airways (from trachea through generation 17). The alveolar region participates in gas exchange, whereas bronchial region in gas conduction. (George et al 2004) This mathematical model describes the NO exchange dynamics in the human lungs and  $F_{E}NO$  at given flow rate is determined by an exponential function:

$$F_{E}NO = C_{aw}NO + (C_{A}NO - C_{aw}NO)e^{-\frac{D_{aw}NO}{V_e}} \quad \text{Eq. 1}$$

Where  $C_{aw}NO$  is NO concentration in the bronchial wall (ppb),  $C_{A}NO$  is NO concentration in alveolar region (ppb),  $D_{aw}NO$  is NO diffusion capacity of the airway wall (pl/s/ppb) and  $V_e$  is flow rate of exhalation (ml/s).

Measuring  $F_{E}NO$  at several flow rates (extended  $F_{E}NO$  measurement) enables the calculation of flow independent NO-parameters. Several mathematical methods based on the two-compartment model's equation and its approximations have been introduced for this purpose. Some of the models use both high and low flow rates and utilize the original non-linear equation or its modifications (Hogman et al 2002, Silkoff et al 2000, Eckel et al 2014) to solve all the flow independent NO-parameters ( $C_{A}NO$ ,  $C_{aw}NO$  and  $D_{aw}NO$ ), while others (Tsoukias et al 2001, Pietropaoli et al 1999) use only medium and high flow rates and a linear approximation of the non-linear equation. To solve  $C_{A}NO$  and  $J_{aw}NO$ , Tsoukias et al used a linear approximation (T&G-method):

$$V_{NO} = C_{A}NO * V_e + J_{aw}NO \quad \text{Eq. 2}$$

where  $V_{NO}$  is the total NO output to the exhaled breath ( $V_{NO} = F_{E}NO * V_e$ ) and  $J_{aw}NO$  is bronchial flux of NO from bronchial wall to luminal air ( $J_{aw}NO = D_{aw}NO * (C_{aw}NO - C_{A}NO)$ ). There are also some models that take into account the increasing cross-sectional area of the airways towards the periphery and possible back diffusion of NO from conducting airways towards alveoli (so called TMAD (trumpet model, axial diffusion) correction) (Condorelli et al 2007). One method differs from the others by utilizing only one blow with dynamically changing flow rate and is able of estimating all the NO-parameters (Tsoukias et al 1998). These methods have been previously reviewed in detail by George and colleagues (George et al 2004).

## Current knowledge

Both increased and normal levels of flow independent NO-parameters have been reported in subjects with asthma.  $J_{aw}NO$  is found increased in most of published studies (Lehtimaki et al 2000, Kanazawa et al 2010, Shimoda et al 2016, Keen et al 2011, Pedroletti et al 2003), but both increased (Kanazawa et al 2010, Shimoda et al 2016) and normal (Lehtimaki et al 2000, Keen et al 2011) levels of  $C_A NO$  have been reported in subjects with asthma.  $D_{aw}NO$  and  $C_{aw}NO$  are rarely reported in the current literature and both increased (Pedroletti et al 2003, Kim et al 2017) and normal (Keen et al 2011, Kim et al 2017) levels are reported. Some studies have focused on glucocorticoid-naïve asthma while others have measured NO parameters in glucocorticoid-treated asthma. Although flow-independent NO parameters have been studied in asthma for almost two decades with several narrative reviews published (Hogman and Merilainen 2007, Garcia-Rio et al 2011, Hogman 2012), extended  $F_E NO$ -measurement in asthma still lacks a systematic review and meta-analysis of the key results.

## Aims of the study

The aim of the study was to conduct a systematic review and meta-analysis to clarify the difference in NO-parameters between subjects with asthma and healthy population. We divided the asthmatics into two groups according to whether they do or do not use glucocorticoids (inhaled glucocorticoids (ICS) or oral glucocorticoids (OCS)), as these are known to cause a significant change in the NO-parameters (Fritscher et al 2009, Spears et al 2011, Van Muylem et al 2010, Leivo-Korpela et al 2011).

## Methods

### Protocol and registration

This study was part of a wider project in which we are conducting systematic reviews on technical aspects and clinical applicability of the multiple flow rate  $F_E NO$ -measurement. The study protocol was included in the International Prospective Register of Systematic Reviews (PROSPERO, [www.crd.york.ac.uk/prospero/](http://www.crd.york.ac.uk/prospero/)) (ID = CRD42017067968). This study focuses on extended  $F_E NO$ -measurement in asthma and was conducted according to the PRISMA-statement (Liberati et al 2009) where appropriate.

### Eligibility criteria

All original journal articles including at least one calculated NO-parameter in both asthmatics and healthy controls were included. Only studies with on-line measurements and human subjects were included (e.g. modellings and animal studies excluded). No other criteria were set according to the type of trial, participants, outcomes or interventions (PICO-elements) in the search stage.

### Search strategy

The purpose of the original search strategy was to identify all studies that reported NO-parameter(s) for one or more subject group. The search was performed using the following databases: Ovid Medline, Scopus, Web of Science and Cochrane Library. The search was executed by using the following search terms:

- a. "alveol\* NO" or "alveol\* nitric oxide" or  $Calv$  or  $C_A NO$
- b. "bronchial nitric oxide" or "bronchial NO" or  $J_{aw}NO$  or  $C_{aw}NO$  or  $D_{aw}NO$
- c. "nitric oxide" and (exhal\* or expir\*)

- d. Citing articles to the first article describing the two-compartment model (Tsoukias and George 1998)
- e. (a or b) and c or d

The search was performed based on topic (TS=topic) in Web of Science and title, abstract and keywords in Scopus and Cochrane Library. In Ovid Medline, advanced search was used (database: “Ovid MEDLINE(R) Epub Ahead of Print, In-Process & Other Non-Indexed Citations, Ovid MEDLINE(R) Daily, Ovid MEDLINE and Versions(R)”). No language or time scale restrictions were used. The search was run on 25.3.2019.

### Study selection

Before study selection, exact duplicates were excluded by using the RefWorks built-in tools and close duplicates were manually inspected whether they were duplicates or not. Then, the study eligibility assessment was performed first according to the title and abstract. If at least one of the NO-parameters or multiple flow rates were mentioned, or it was suspected that the full text would, full text was included in further eligibility assessment. More precise exclusion criteria according to the full text assessment are listed in the flow chart (Figure 1). Study selection process was carried out by the authors independently and all disagreements were resolved in consensus.

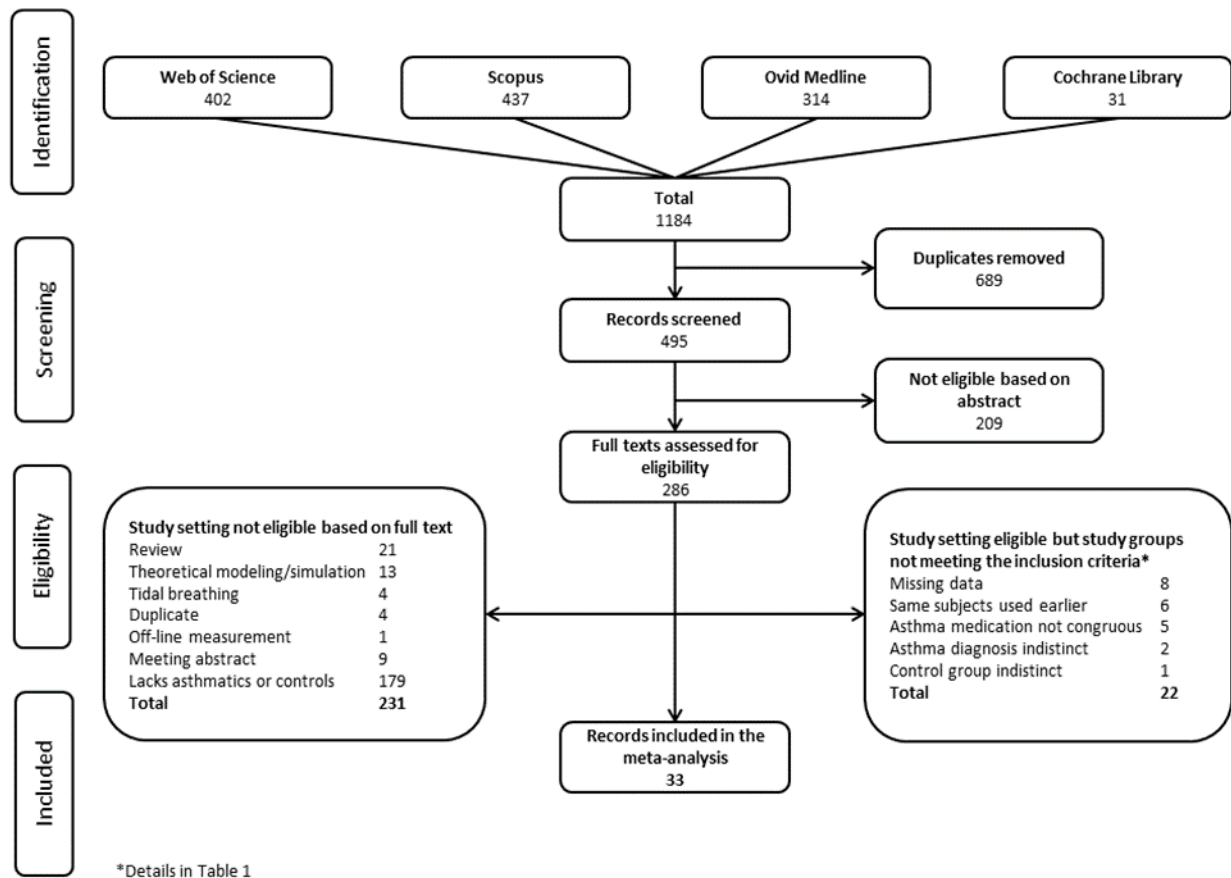


Figure 1. Flow chart of the study selection.

## Data extraction

Data extraction was performed by one researcher and the extraction was completed in duplicate after the first extraction. The extracted data was also checked by the other researcher independently.

Double publication was suspected if the NO-parameters and subject numbers were the same between different studies from the same group or prior usage of study population was mentioned in the article. If the same data had been used in more than one study, only the first publication was included in the analysis. Same controls were allowed but the asthmatic subjects had to be different in every study group. If the data needed were not included in the published paper or it was in an inappropriate form for the meta-analysis, the corresponding author was contacted via email. If no reply was received, study was excluded from the meta-analysis or was included only by applicable parts.

## Data items

The following data items were extracted from each study: 1) reference, 2) participant demographics (number, age, severity/type/control of asthma as described, criteria of asthma diagnosis, FEV<sub>1</sub> % predicted, smoking habits, atopy/allergy as reported, use of inhaled or systemic glucocorticoids), 3) NO-measurement (used flow rates, used mathematical method and NO-parameter values) and 4) funding and conflicts of interest.

In some studies, the NO-parameters had been calculated by using multiple mathematical methods. Results from only one method per study were included in this meta-analysis to reduce risk of bias caused by usage of different methods that are known to yield varying results and to avoid overweighting of individual studies in the meta-analysis. If a non-linear method was used, results from that were extracted and used in the meta-analysis since these methods can be used to calculate all the NO-parameters. If only linear methods had been used, T&G was preferred since it is the most widely used method (T&G with TMAD-correction and Pietropaoli-method were used only if other were not presented).

In most studies, the NO-parameters were reported in form of mean  $\pm$  SD (standard deviation) or mean  $\pm$  SEM (standard error of mean). Results reported as SEM were converted into SD by the following formula:

$$SD = \sqrt{n} * SEM \quad (\text{Higgins and Green 2011})$$

If the parameters were reported in another form (i.e. median and range, median and interquartile range (IQR) or mean and 95 % confidence interval) they were converted into the form of mean  $\pm$  SD as it was necessary for the meta-analysis. Parameters reported in the form of median – 1<sup>st</sup> and 3<sup>rd</sup> interquartile range (IQR) or median and range were converted into mean  $\pm$  SD according to Wan et al (Wan et al 2014). Results in the form of mean and its CI 95 % were manually converted into mean  $\pm$  SEM if both upper and lower confidence intervals were within the same distance from the mean. Data conversion was based on the equation:

$$SD = \sqrt{n} * (\text{CI upper} - \text{CI lower}) / 3.92 \quad (\text{Higgins and Green 2011})$$

In some cases, mean  $\pm$  SD was calculated from scratch if the authors reported only individual subjects' NO-parameters. In these cases, a test of normality was deployed (Shapiro-Wilk) and normality of the parameters was tested. Non-normally distributed parameters were treated as other data conversions in the analyses. In other cases, the authors were contacted via email and asked to provide us with a suitable form of data to be used in the meta-analysis. Values of J<sub>aw</sub>NO were converted into pl/s if they were reported in another form (e.g. nl/min, nl/s). As these conversions may cause bias, the meta-analysis was also

performed without converted results by including only studies that originally reported results as mean  $\pm$  SD or SEM and excluding those that needed to be converted.

### Risk of bias in individual studies

As there were no randomized controlled studies, we could not conduct the risk of bias assessment as generally recommended for systematic reviews. The risk of bias was assessed descriptively based on methodological aspects of the extended  $F_{E}NO$ -measurement, smoking habits of the study subjects, basis of asthma diagnosis, number of subjects and conflicts of interest in a table but these had no impact on the final analyses.

### Risk of interstudy bias

The risk of publication bias across studies was first assessed by estimating funnel plots' asymmetry. As visual estimation of funnel plot's symmetry may be subjective, symmetry was also estimated formally by performing Egger's test. Funnel plots were drawn only for  $C_{A}NO$  and  $J_{aw}NO$ , since sample sizes of other parameters were too low. Heterogeneity was assessed by calculating  $I^2$ -statistics. Funnel plots were drawn also for studies using only T&G-method to reduce the risk of bias caused by differences between mathematical methods in funnel plot asymmetry.

### Characteristics of the study groups

#### *Asthmatics*

Asthmatics were considered glucocorticoid-naïve, if at least 2/3 of the subjects in the group were not on oral or inhaled glucocorticoids for at least 4 weeks. Asthmatics were considered glucocorticoid-treated if at least 2/3 of the subjects were on regular ICS or OCS. Atopy and allergy, short description of asthma as reported by the study authors, smoking habits and FEV1 % predicted are reported for each group in the table but these had no impact on the meta-analysis. Groups with asthma exacerbations were excluded from the meta-analysis.

#### *Controls*

Both glucocorticoid-naïve and glucocorticoid-treated asthmatic groups had their own control groups from the respective studies. If more than one control group was present in a study, "the healthiest" was chosen for the meta-analysis (e.g. group without allergy or rhinitis). Otherwise the control group was expected to be healthy as the authors had reported.

### Summary measures and synthesis of results

Arithmetic mean and 95 % confidence interval of NO-parameters were calculated for each study group. Mean difference with 95 % confidence interval between asthmatic and healthy subjects was calculated for each NO-parameter within each study.

For each individual study, a raw mean difference (MD) between the NO-parameters of asthmatic and healthy subjects ( $MD = \text{asthma} - \text{healthy}$ ) was calculated and inverse variance weighting was deployed to calculate the weights for individual studies. The meta-analysis was conducted by computing mean differences and summary estimate with a random-effects model using DerSimonian-Laird -approach. Random-effects model was chosen because of considerable heterogeneity between different studies and we assumed that the included studies represented a random sample from a larger population and there are multiple confounding factors among studies. Mean difference and 95 % confidence interval was calculated for each side effect using the random-effect model. The mean difference of NO-parameters compared to healthy controls was calculated separately for asthmatics on glucocorticoids (either inhaled or systemic) and glucocorticoid-naïve asthmatics. We decided not to calculate mean differences between

glucocorticoid-treated and glucocorticoid-naive asthmatics, as the nature of asthma in these groups is probably highly different in severity and duration of the disease.

Meta-analysis and all related statistics were calculated using R version 3.4.3 (R Core Team 2016) and R-package Metafor (Viechtbauer 2010). Metafor-package was deployed to draw all plots present in this review.

## Results

### Study selection

A total of 33 studies were included in the meta-analysis. Overall, 1184 studies were identified in the search. Duplicates were removed, and 495 studies were left for eligibility assessment by title and abstract. 209 studies were discarded as they clearly did not meet the eligibility criteria. After this, we were left with 286 studies for full text assessment of eligibility and a total of 231 studies were excluded in this process (the study selection process better described in Figure 1). The remaining studies were searched for asthmatic and healthy control groups and only these were used in this project. In the final phase of study selection, subject groups' applicability was checked, and 22 studies were excluded (the exact causes of exclusions are listed in Table 1). Risk of bias was attempted to be minimized by excluding studies in which information on subjects' diagnoses or medication were too indefinite. There were missing data in 8 studies that could not be received from the study authors.

Table 1. Reasons of exclusion based on study groups' properties and missing data.

<b>Reason of exclusion</b>	<b>Number of studies</b>	<b>Description</b>	<b>References</b>
<b>Missing data</b>	5	NO-parameters not reported in a suitable form for the meta-analysis (e.g. mean $\pm$ log SD, geometric mean).	(Silkoff et al 2000, Brindicci et al 2007b, Williamson et al 2011, Kobayashi et al 2011, Malinovschi et al 2006)
	1	NO-parameters of age-matched control group not reported.	(Gelb et al 2012)
	1	Number of subjects not reported.	(Shorter et al 2011)
	1	Reported data form unclear.	(Brindicci et al 2007a)
<b>Same subjects used earlier</b>	6	The same subjects had been included in an earlier study.	(Leivo-Korpela et al 2011, Lehtimaki et al 2002, Lehtimaki et al 2001, Lehtimaki et al 2005, Linn et al 2013, Eckel et al 2015)
<b>Asthma medication not congruous</b>	5	Asthmatic study group could not be categorized into glucocorticoid-naive or glucocorticoid-treated according to our criteria.	(Bake et al 2014, Hogman et al 2001, Puckett et al 2010, Shin et al 2007, Tufvesson et al 2013)
<b>Asthma diagnosis indistinct</b>	2	Asthma diagnosis was based on questionnaires on asthma and allergy related symptoms, asthma-like symptoms.	(Rosa et al 2011, Knihtila et al 2018)
<b>Control group indistinct</b>	1	Controls had airway hyperresponsiveness and nasal polyposis.	(Gelb et al 2012)
<b>Total</b>	22		

## Study characteristics

*Study design* was cross-sectional in most cases. A few prospective cohort studies were identified but due to our study design we used only the first NO-measurement if  $F_E\text{NO}$ -measurement had been performed multiple times.

*Methods:* A total of 16 studies with glucocorticoid-naive and 27 with glucocorticoid-treated asthma groups were included in the meta-analysis. The most frequently used method in calculating the parameters was T&G (Tsoukias & George (Tsoukias et al 2001), used in 23 studies). Other used methods were HMA (Högman & Meriläinen -algorithm (Hogman et al 2002), 6 studies), T&G with TMAD correction (TMAD: Trumpet Model of Axial Diffusion (Condorelli et al 2007), 2 studies), Silkoff (Silkoff et al 2000) (1 study), Eckel (Eckel et al 2014) (1 study) and Dynamic one flow -method (Tsoukias et al 1998) (1 study). Numerous different flow rate combinations were used in the parameter calculations (Details in e-supplement Table 1).

*Participants:* Overall, 718 glucocorticoid-naive and 1070 glucocorticoid-treated (altogether 1788) asthmatics were included in the studies. They were compared to 1781 and 735 healthy subjects, respectively. Since some studies used the same healthy control group for comparison to glucocorticoid-treated and glucocorticoid-naïve asthmatics, the total number of healthy subjects was not the sum of these figures but 2409.

Glucocorticoid-naive asthmatic group consisted mostly of newly diagnosed and mild asthma. Glucocorticoid-treated asthmatics were a heterogenous group of asthmatics with different disease severities and treatment levels. In some cases, the authors had not described the asthmatics in detail except for the usage of ICS or OCS. Mostly, the groups consisted wholly of glucocorticoid-using asthmatics. For more details, see e-supplement tables 1 and 2.

## NO-parameters in asthma

All NO-parameters were increased in both glucocorticoid-treated and glucocorticoid-naive asthma as compared to respective healthy control groups. Increase was most distinct in  $C_A\text{NO}$  and  $J_{aw}\text{NO}$ .  $D_{aw}\text{NO}$  and  $C_{aw}\text{NO}$  were also increased but increase was more restrained in both asthmatic groups. Results are presented in the Table 2 and Figures 2-5 and e-supplement figures 3-6. Due to marked differences between the two asthma groups, we did not conduct comparison between glucocorticoid-treated and glucocorticoid-naïve asthma, as these groups were highly heterogenous.



Table 2. The NO-parameters of asthmatic and healthy subjects.

<b>Study group</b>	<b>C<sub>A</sub>NO (ppb)</b>	<b>J<sub>aw</sub>NO (pl/s)</b>	<b>C<sub>aw</sub>NO (ppb)</b>	<b>D<sub>aw</sub>NO (pl/s/ppb)</b>
<b>Glucocorticoid-naive asthma</b>				
Number of studies	15	14	3	4
Number of subjects (asthma/control)	797/1865	770/1847	221/383	542/1697
Glucocorticoid-naive asthmatics	3.58 (2.98–4.18)	2220 (1903–2538)	198 (56–340)	16.4 (10.6–22.3)
Healthy controls	2.32 (1.94–2.70)	727 (644–811)	121 (40–202)	14.5 (6.0–23.1)
MD*	1.19 (0.73–1.65) ‡	1418 (1102–1734) ‡	34 (14–54) †	2.3 (0.8–3.8) †
I <sup>2</sup> (Total heterogeneity)	88.73 %	78.77 %	28.24 %	43.17 %
<b>Glucocorticoid-treated asthma</b>				
Number of studies	20	18	4	4
Number of subjects (asthma/control)	1025/592	765/513	89/114	89/114
Glucocorticoid treated asthmatics	4.35 (3.76–4.95)	2077 (1666–2487)	197 (134–260)	25.9 (15.0–36.9)
Healthy controls	2.63 (2.29–2.96)	706 (615–797)	119 (74–164)	10.7 (6.3–15.1)
MD	1.53 (1.03–2.02) ‡	1314 (933–1695) ‡	77 (38–116) ‡	13.8 (5.6–22.01) †
I <sup>2</sup> (Total heterogeneity)	88.92 %	94.93 %	38.92 %	92.34 %

Data presented in the form of mean (CI 95%), \* MD = Mean difference, healthy controls – asthmatics, † p-value ≤ 0.05, ‡ p-value < 0.001

### Healthy subjects vs steroid naive asthmatics JawNO

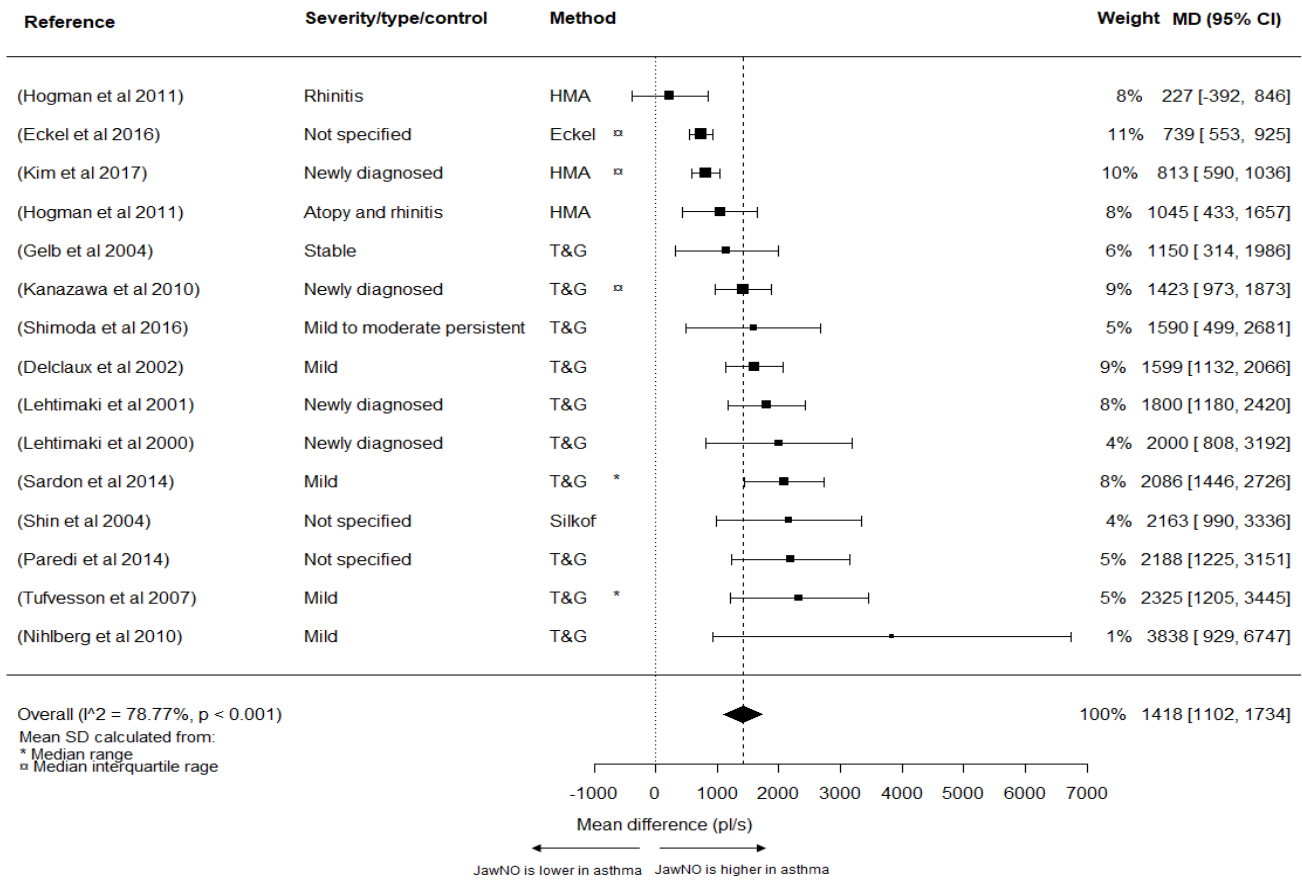


Figure 2. Difference in  $J_{aw}NO$  between healthy controls and glucocorticoid-naive asthmatics.

### Healthy subjects vs steroid naive asthmatics CaNO

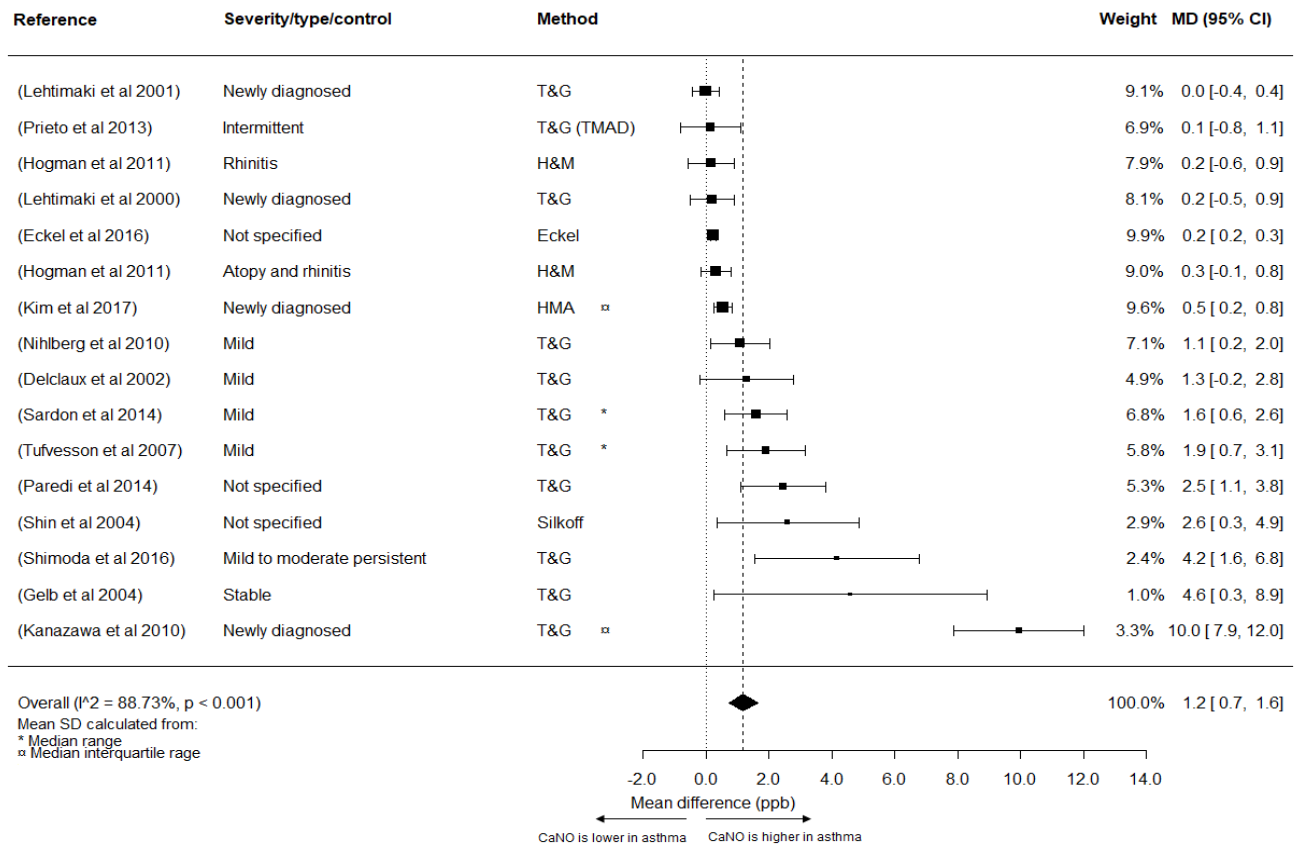


Figure 3. Difference in  $C_{a}NO$  between healthy controls and glucocorticoid-naive asthmatics.

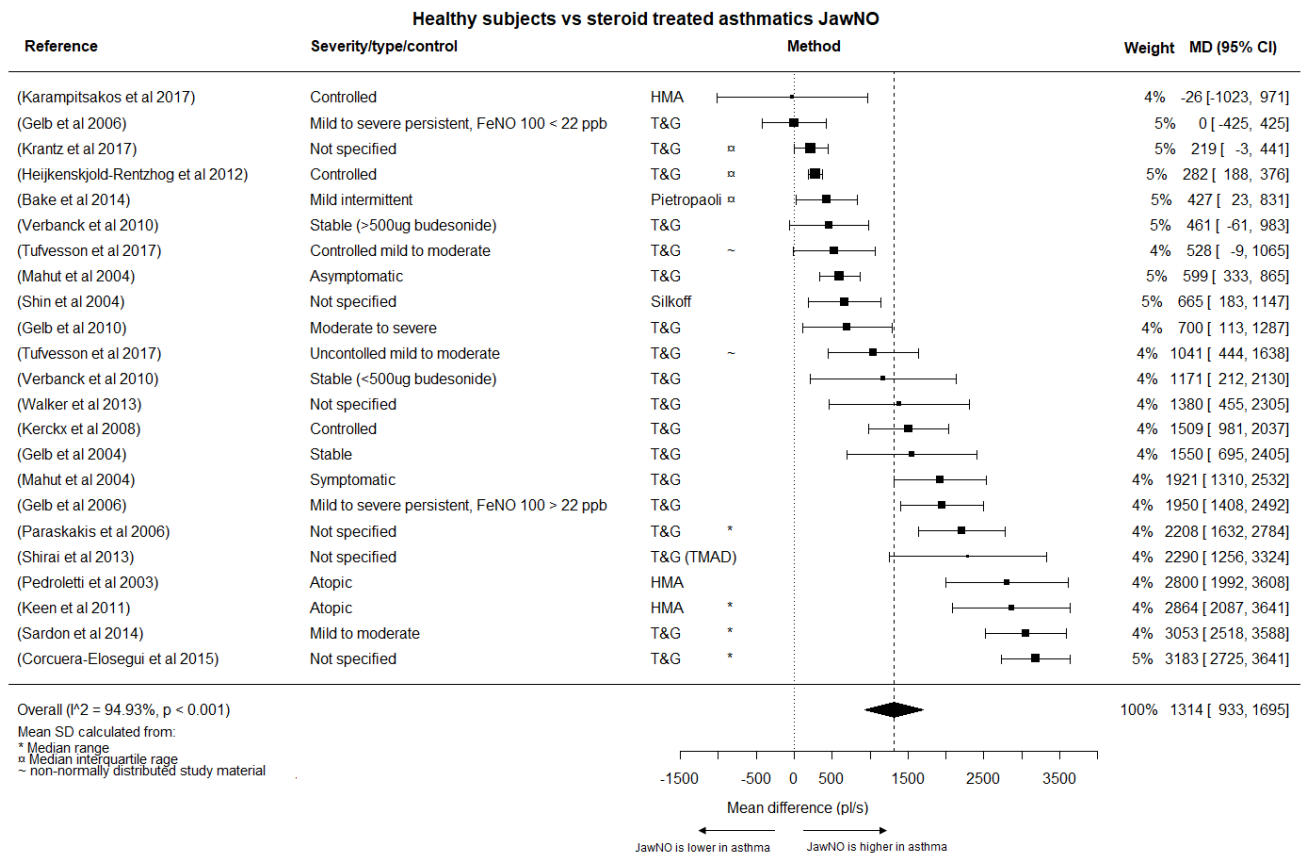


Figure 4. Difference in  $J_{aw}NO$  between healthy controls and glucocorticoid-treated asthmatics.

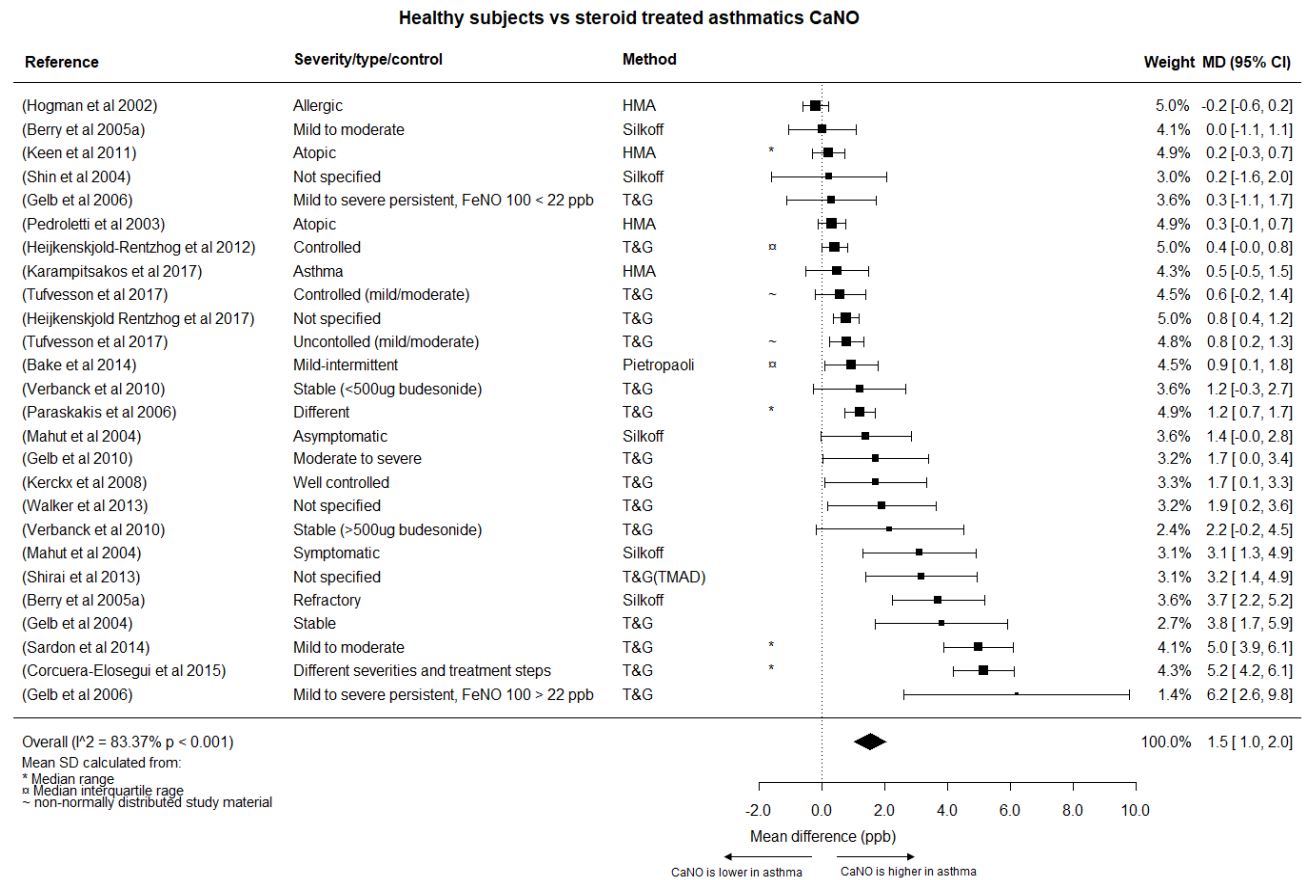


Figure 5. Difference in  $C_{a}NO$  between healthy controls and glucocorticoid-treated asthmatics.

To check for the possible confounding effect of data conversion, the meta-analysis was also performed after exclusion of all studies with data conversions or known non-normally distributed NO-parameters. After exclusion of these studies, the main results remained the same. Both  $C_{A}NO$  and  $J_{aw}NO$  were higher in both asthma groups as compared to healthy controls. The number of studies reporting  $D_{aw}NO$  and  $C_{aw}NO$  was small (2 and 3 studies) and although the results tended to be the same after exclusion of studies with data conversion, there was no more statistically significant difference between healthy controls' and glucocorticoid-naïve asthmatics' in  $D_{aw}NO$  and  $C_{aw}NO$ . The effect of data-conversions was also evaluated by combining all healthy controls in single group and no significant differences were noticed in  $C_{A}NO$  or  $J_{aw}NO$  when no conversion was used as intercept (results not shown).

In addition, to assess possible effect of mathematical method on results, we combined all healthy control groups and investigated the possible impact of estimation methods on results by setting the used method as a moderator and using T&G as intercept. However, we did not find statistically significant differences. T&G (TMAD) yielded higher estimates for  $J_{aw}NO$ , but this result should be interpreted cautiously as only one study had estimated  $J_{aw}NO$  using T&G (TMAD).

### Risk of bias across studies

A strong level of heterogeneity was observed in the mean differences between healthy and asthmatic subjects ( $I^2$  statistics are presented in Figures 2-5 and e-supplement figures 3-6 and Table 2). Most heterogenous parameters in this regard were  $C_{A}NO$  and  $J_{aw}NO$  in both asthmatic groups, whereas distinctly least heterogenous was  $C_{aw}NO$ .  $D_{aw}NO$  was more heterogenous in glucocorticoid-treated asthma (92.34 %) compared to glucocorticoid-naïve (43.17 %).  $I^2$  statistics was calculated for the mean differences with and without data conversions. The  $I^2$  values for  $C_{A}NO$  and  $J_{aw}NO$  seemed to be lower after exclusion of studies with data conversions, possibly indicating bias caused by the data-conversions.  $D_{aw}NO$  and  $C_{aw}NO$  yielded higher values of  $I^2$  in studies without data-conversions, except  $D_{aw}NO$  in glucocorticoid-treated asthma.

### Heterogeneity and publication bias

Interstudy heterogeneity was further investigated by funnel plots (e-supplement figure 1). However, funnel plots were drawn only for  $C_{A}NO$  and  $J_{aw}NO$ , as minimum of 10 studies is recommended to be included in a funnel plot (Sterne et al 2011). All funnel plots were highly asymmetrical according to the Egger's test of asymmetry: glucocorticoid-treated asthmatics ( $C_{A}NO$ :  $z = 3.28$ ,  $p < 0.01$ ;  $J_{aw}NO$ :  $z = 4.01$ ,  $p < 0.001$ ) and glucocorticoid-naïve ( $C_{A}NO$ :  $z = 3.18$ ,  $p < 0.001$ ;  $J_{aw}NO$ :  $z = 4.13$ ,  $p < 0.001$ ). After exclusion of other mathematical methods than T&G, Egger's test showed little less asymmetry: glucocorticoid-treated asthmatics ( $C_{A}NO$ :  $z = 2.25$ ,  $p = 0.04$ ;  $J_{aw}NO$ :  $z = 3.42$ ,  $p < 0.01$ ) and glucocorticoid-naïve ( $C_{A}NO$ :  $z = 3.64$ ,  $p < 0.01$ ;  $J_{aw}NO$ :  $z = 2.22$ ,  $p = 0.06$ ) (e-supplement figure 2).

## Discussion

### Summary of evidence

To our knowledge, this is the first systematic review and meta-analysis addressing the flow independent NO-parameters in asthma. This meta-analysis showed rather concurrently that  $C_{A}NO$  and  $J_{aw}NO$  are increased in asthma. We consider this evidence quite strong since sample size was relatively large and the included studies showed similar results. However, there are many confounding factors (e.g. age, atopy, technical aspects) that could not be taken in account in this meta-analysis. Evidence on  $C_{aw}NO$  and  $D_{aw}NO$  remains still quite weak as studies had rarely reported them. More knowledge should be gained on these parameters to draw better conclusions on their behavior in asthma and to evaluate their clinical significance.

### *NO parameters of the gas conducting region ( $C_{awNO}$ , $D_{awNO}$ , $J_{awNO}$ )*

$C_{awNO}$  describes the concentration of NO in the bronchial wall tissue. The concentration is determined by the balance between the production and elimination rates of NO in the bronchial wall mucosa. Production is thought to represent activity of nitric oxide synthases, while elimination is either diffusion of NO from mucosa to luminal air or blood circulation, or consumption in chemical reactions.  $D_{awNO}$ , on the other hand, describes the diffusion capacity of NO between bronchial mucosa and luminal air.  $D_{awNO}$  is dependent on both physical diffusivity of NO between mucosa and air, and size of the NO-producing surface area.  $D_{awNO}$  may thus be increased due to physical changes in the mucosa improving the physical diffusivity of NO or if the surface area producing NO increases e.g. by spreading of the asthmatic inflammation from central airways towards peripheral airways. On the other hand, also bronchial obstruction may affect  $D_{awNO}$ , as bronchial obstruction decreases not only the cross-sectional area of airways but also the total mucosal surface area of the airways that is in contact with luminal air. This is one theory to explain decreased  $D_{awNO}$ , and thus decreased  $J_{awNO}$  and  $F_E NO$ , in state of obstruction (Verbanck et al 2008).

In this meta-analysis,  $D_{awNO}$  was elevated in both glucocorticoid-naïve and glucocorticoid-treated asthmatics. The exact mechanism of increased  $D_{awNO}$  in asthma is not known, but as discussed above, it may be related to either spreading of the NO production towards peripheral airways or to physical changes in mucosa improving the actual diffusivity of NO. Both these are plausible explanations, as  $D_{awNO}$  has been shown to be increased also in atopy and allergic rhinitis (Makinen et al 2009, Hogman et al 2011) and several physical changes in the airways have been documented in asthma as a consequence of airway remodeling (e.g. epithelial shedding, goblet cell hyperplasia, basal membrane thickening, subepithelial fibrosis and smooth muscle hypertrophy) (Fehrenbach et al 2017). Interestingly glucocorticoid-treatment does not seem to affect  $D_{awNO}$  (Silkoff et al 2000, Hogman and Merilainen 2013), suggesting that whatever the mechanism behind increased  $D_{awNO}$  in asthma is, it is not sensitive to glucocorticoids.

$C_{awNO}$  was mildly elevated in both asthmatic groups. However, no strong conclusions should be drawn based on these results as the number of studies reporting  $C_{awNO}$  was extremely low. We decided not to compare the two asthmatic groups as these groups had marked differences in the severity and duration of asthma in addition to the low number of studies reporting  $C_{awNO}$ . In theory, the anti-inflammatory medication can restrain the inflammation in the bronchial wall and thus lower  $C_{awNO}$  and  $J_{awNO}$ , leaving  $C_{awNO}$  of glucocorticoid-naïve asthmatics higher. Inhaled glucocorticoids are known to suppress the expression of iNOS, which is responsible for the increased levels of NO in inflammation (Korhonen et al 2002). Meanwhile, the possible tissue changes remain despite of the anti-inflammatory medication and  $D_{awNO}$  remains unchanged. Indeed, it has been shown that ICS lower  $C_{awNO}$  but not  $D_{awNO}$  in acute asthma (Silkoff et al 2000, Hogman and Merilainen 2013). The current literature is still deficient to draw exact conclusions on the behavior of  $D_{awNO}$  in asthma and glucocorticoid-treatment and more research is required. This meta-analysis showed a clear trend that  $J_{awNO}$  is elevated in both glucocorticoid-treated and naïve asthma. The increase in  $J_{awNO}$  is probably due to both increase of  $C_{awNO}$  and  $D_{awNO}$ .

### *Alveolar NO ( $C_A NO$ )*

Alveolar NO was found to be elevated in both asthmatic groups, possibly suggesting inflammation in the distal lung. Inflammation in the distal lung has been demonstrated in severe asthma using tissue biopsies (Kraft et al 1996) and post-mortem tissues (Mauad et al 2004).  $C_A NO$  has been investigated as a possible non-invasive marker of distal lung inflammation and elevated  $C_A NO$  levels are noticed to be linked to severe, nocturnal and symptomatic asthma in some studies (Lehtimaki et al 2002, Lehtimaki et al 2005, van Veen et al 2006). However, there are several different mechanisms that may cause increase in  $C_A NO$ .

In the original two-compartment model airway generations from 18 and beyond were included in the alveolar compartment (Tsoukias and George 1998). Thus, the small respiratory bronchioles, which are the transition zone of conducting airways to gas transfer zone, are included in alveolar compartment. Inflammatory activity in these small airways might therefore directly increase  $C_{A}NO$ . Other possible mechanisms are increased back-diffusion of NO from larger conducting airways or decreased uptake of NO to pulmonary circulation due to impaired diffusion of NO from alveoli to pulmonary capillaries.

In addition to possible marker of peripheral inflammation,  $C_{A}NO$  has also been suggested as a marker of peripheral airway obstruction as  $C_{A}NO$  has been noticed to positively correlate with obstruction (Kobayashi et al 2011, Fujisawa et al 2013, Barbinova et al 2013). The effect of inhaled and systemic glucocorticoids on  $C_{A}NO$  is not clear, as there are conflicting results. Some studies have noticed decreasing of  $C_{A}NO$  with systemic glucocorticoids (Silkoff et al 2000, Van Muylem et al 2010, Gelb et al 2004). However, fine particle ICS or systemic glucocorticoids had no significant effect in reducing  $C_{A}NO$  in severe asthma in a randomized controlled study (Williamson et al 2013). The idea of treating severe asthma with systemic glucocorticoids based on elevated  $C_{A}NO$  is that ICS is not able to reach the smallest conducting airways.

#### *Axial back-diffusion of NO and TMAD*

It has been hypothesized that the conventional methods based on the two-compartment model overestimate alveolar NO and underestimate bronchial NO due to axial back-diffusion of NO from the more NO-rich conducting region into the alveolar region. The two-compartment model assumes the conducting region as an even, cylinder shaped tube, neglecting the increasing total cross-sectional area of the bronchial lumen towards peripheral airways. As the total cross-sectional area of the bronchial lumen increases with every new generation of bronchi, velocity of the air flow decreases simultaneously. The decrease in air flow velocity is believed to be significant to allow axial back-diffusion of NO from the bronchial region into the alveolar region in the distal lung.

The axial back-diffusion can lead to underestimation of  $J_{aw}NO$  and  $D_{aw}NO$  as bronchial NO diffuses from conducting region into alveolar region (Shin and George 2002). However, obstruction is believed to inhibit the back-diffusion, making the underestimation of bronchial NO greater in subjects without obstruction (Heijkenskjold-Rentzhog et al 2014). This may also partly explain why  $D_{aw}NO$  and  $J_{aw}NO$  were elevated in asthma as compared to healthy subjects: the back-diffusion of NO is higher in healthy subjects, leading to greater underestimation of  $D_{aw}NO$  and  $J_{aw}NO$  relative to asthmatics with obstruction.

Condorelli et al. introduced the trumpet model of axial back-diffusion (TMAD) of NO to consider the axial back-diffusion by applying correction factors to the NO-parameters calculated by the conventional two-compartment model (Condorelli et al 2007). The trumpet model assumes the conducting region as trumpet-shaped instead of even cylinder-shaped, taking account the increasing total cross-sectional area of bronchial lumen in the lung periphery. However, the TMAD-correction applies only to subjects with no obstruction as peripheral obstruction can reduce the axial back-diffusion, possibly causing over-correction (Heijkenskjold-Rentzhog et al 2014). For this reason, we did not include TMAD-corrected results in the meta-analysis, unless it was the only method used in a study (TMAD was the only method in only two included studies).

#### *Possible clinical applications in asthma*

The NO-parameters could be used together with other methods in the diagnosis, management and phenotyping of asthma. They could also be utilized in the prediction and follow-up of treatment response to ICS. For instance, knowing the behavior of  $D_{aw}NO$  is important if one is to plan glucocorticoid treatment according to  $F_{E}NO$  measurement, since  $D_{aw}NO$  is elevated in asthma and glucocorticoid

treatment seems not to greatly influence it. For this reason, the target  $F_{E}NO$ -values for treated asthmatics may not be entirely normal values but set to a higher level keeping in mind the elevated  $D_{aw}NO$  and hence elevated  $F_{E}NO$  and  $J_{aw}NO$  regardless of normalized  $C_{aw}NO$  (Hogman and Merilainen 2007).  $C_{aw}NO$  on the other hand would be a better indicator for success of anti-inflammatory treatment. Treatment response and dosing could be titrated for each individual according to decrease in  $C_{aw}NO$ . (Hogman et al 2017) Measurement of  $C_{A}NO$  offers interesting possibilities for research purposes and it has been investigated as a possible marker of distal lung inflammation and peripheral obstruction. Systemic glucocorticoids or other systemically administered drugs could be given according to the inflammation in the distal airways, indicated by  $C_{A}NO$ . However, results about correlation with disease severity and effect of systemic glucocorticoid treatment are still somewhat conflicting. Further research should be done using standardized methodology to calculate  $C_{A}NO$  and appropriate study protocols.

## Strengths and limitations

One strength of this study was the relatively large sample size. There were 33 included studies with altogether 4304 subjects that provided results from many different study groups. Our search strategy was also rather comprehensive as its original purpose was to identify all studies using extended  $F_{E}NO$ -measurement.

Despite of the rather reliable results regarding  $C_{A}NO$  and  $J_{aw}NO$  in asthma, the current knowledge has some considerable limitations. There is a high risk of inter-study bias, as multiple potential and known confounding factors were identified and the results were highly heterogenous. Methodological aspects included different flow rates and mathematical methods, which are known to produce different results (Chladkova et al 2012, Karvonen et al 2017, Roy et al 2007). Also, other possible methodological issues may cause bias, as extended  $F_{E}NO$ -measurement lacks standardization (e.g. analyzer calibration, time of measurements taking account possible diurnal variation).

One possible source of heterogeneity is the heterogeneity within the study subjects themselves since asthma is a heterogenous disease. In this meta-analysis, asthmatics were grouped into two groups only according to glucocorticoid usage. Authors had described the asthma type, severity and control on different basis and the diagnosis of asthma had been made in different ways. For these reasons we chose not to compare the two asthmatic groups by a meta-analysis, as there are expected to be too many confounding factors. It would have been interesting to group the asthmatics according to the inflammation type (e.g. eosinophilic vs non-eosinophilic or type 2 high vs low) but this was only seldom reported, and study groups were heterogenous regarding the inflammation type.

Some subjects were smokers or former smokers and smoking was not always reported. This may affect the results since active smoking is reported to decrease  $F_{E}NO$  in asthma (Rutgers et al 1998, Verleden et al 1999). Some subjects may have been exposed passively to cigarette smoke and this may have affected the results as cigarette smoke is known to lower  $F_{E}NO$  in healthy and asthmatic subjects (Jacinto et al 2017). Age and height are also known to influence the NO-parameters and we could not include these in the analyses (Hogman et al 2017). In addition, glucocorticoid treatment was dichotomized, and we could not take account the dose of asthma medication. Also, some studies had asthmatic subject groups that in which only some of the subjects used inhaled corticosteroids (in 4 glucocorticoid-naïve and 5 glucocorticoid-treated groups some of the subjects used ICS or other asthma control medication). Some authors were minority shareholders and had received gifts (e.g. NO-analyzers) or grants from NO-analyzer manufacturers but these studies seemed not to differ from studies with no reported conflicts of interests.

Increased  $F_{E}NO$ -levels are associated with eosinophilic inflammation and are positively correlated with sputum eosinophil count (Berry et al 2005b). Eosinophilic asthma can be either  $T_{H2}$ -cell driven allergic asthma or non-allergic eosinophilic asthma (Wenzel 2012). In both these phenotypes the link between  $F_{E}NO$  and eosinophilia are the type 2 cytokines. IL-5 is the most important cytokine leading to eosinophilia while IL-13 drives iNOS expression and NO synthesis in the epithelium (Chibana et al 2008, Brusselle et al 2013). Ideally studies on  $F_{E}NO$  should focus only on these two eosinophilic phenotypes of asthma, but since the subjects were mainly not phenotyped in this regard in the original studies, the inclusion of non-eosinophilic subjects likely decreases differences between asthmatic and healthy subjects in the current analysis.

Heterogeneity and inter-study bias were also investigated by using funnel plots, which were highly asymmetrical. Funnel plot's weakness is that it may give a false impression of publication bias if high precision studies differ from low precision studies in effect size (e.g. studies with small sample size yield greater mean differences). Other explanation for the asymmetry could be systematic bias derived from using different mathematical models, analyzers and their calibration, flow rates or different study populations. We further investigated the asymmetry of the funnel plots by plotting only studies using T&G-method, but significant asymmetry remained in the funnel plots. One possible explanation for the funnel plots' asymmetry can be publication bias. Shape of glucocorticoid-naive asthmatics' funnel plots could be explained with publication bias if studies with smaller differences or no differences would have been left unpublished. However, publication bias is only one possible explanation and true heterogeneity between studies could be considered a more plausible explanation (i.e. high precision studies differ from low precision studies in effect size). Glucocorticoid-treated asthmatics' funnel plots seemed more randomly distributed and no clear pattern was observed.

Our study design has also some limitations. Some studies had more than one asthmatic group. We decided to include all study groups if they consisted of different subjects. However, this caused those studies with more than one asthmatic group to gain more total weighting in the meta-analysis than if there was only one group per study. In addition, as the meta-analysis required the results as mean  $\pm$  SD, we had to convert results reported in other forms in order to include these studies in the meta-analysis. It has been noticed that  $J_{aw}NO$  is often right skewed and results were sometimes reported in other form than mean  $\pm$  SD. Optimally, the skewed NO-parameters should have been converted into e.g. geometric mean and logSD for the meta-analysis. However, it is not possible to convert the data into this form without the original data. We could have asked these data from the authors but as we noticed, response rate was extremely low and most studies had reported the NO-parameters as mean  $\pm$  SD. This added bias, as to some degree non-normally distributed data was artificially made normally distributed. However, we decided to include these studies in the meta-analysis as distributions of biological parameters are often skewed to the right, especially in diseased populations. We hypothesized that excluding all non-normally distributed results would underestimate the differences between healthy and diseased in the meta-analysis. However, converting skewed distributions to normal distribution is a rather crude approximation and we calculated the results also without data conversions. However, this might have excluded the studies that reported their results in more appropriate form than the studies reporting results as mean  $\pm$  SD as  $J_{aw}NO$  especially is noticed to be right-skewed. Ultimately conversions seemed not to have substantial impact on the overall results and the effect of data-conversion was tested in healthy controls with no statistically significant differences. Also, a minor source of error may spring out from that some studies had to be excluded from the meta-analysis as missing data could not be obtained from authors.



## Future research

Future research should focus on standardization of the multiple flow NO-measurement. NO-parameters have in theory promising applications in asthma and other diseases, but the current literature on their applicability is still quite conflicting and heterogenous. In order to investigate the utility of NO-parameters in asthma, the impact of different methodological issues on the results must be eliminated. Future researchers should also use the non-linear methods and report all NO-parameters to gain more knowledge on  $C_{aw}NO$  and  $D_{aw}NO$ .

## Conclusions

In summary, this meta-analysis showed a significantly elevated level of all NO-parameters in both glucocorticoid-treated and naive asthmatics as compared to healthy subjects. Due to only few studies reporting  $C_{aw}NO$  or  $D_{aw}NO$ , the evidence on these NO-parameters is still quite feeble. More research should be put on these NO-parameters and standardization of the extended  $F_{E}NO$ -measurement if they are to be taken into clinical practice.

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## Conflicts of interest

We declare no conflicts of interest.

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