Nitric Oxide Exchange in Central and Peripheral Airways

Determinants in Health and Respiratory Disease

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Abstract

Background: Exhaled nitric oxide (NO) is a marker of eosinophilic steroid-sensitive inflammation in the airways of patients with respiratory disease. Moreover, information about the localization of inflammation in the respiratory tree is obtained by estimates of bronchial and alveolar contributions to exhaled NO.

Aims: The main aim of this thesis was to identify the determinants of exhaled NO, as well as determinants of bronchial and alveolar contributions to exhaled NO in health and disease. Smoking history, degree of IgE sensitization and effects of modulating the pharyngo-oral tract production of NO were specifically studied in this context. Other specific aims were to determine the association of exhaled NO with the presence of asthma and pulmonary hypertension (PH).

Methods: Both population-based studies and experimental studies have been performed within the frame of the thesis. The population-based studies are based on data from the European Community Respiratory Health Survey II. NO measurements at several exhalation flow rates were performed in order to estimate alveolar and bronchial contributions to exhaled NO.

Results: Both current and previous smoking were associated with decreased exhaled NO and bronchial NO flux levels. Alveolar NO concentrations were decreased in current smokers. The degree of IgE sensitization was positively related to the levels of exhaled NO and its bronchial contribution. Exhaled NO appeared to be a more specific marker of allergic inflammation than of rhinitis or asthma. Both allergic and non-allergic asthma were associated with increased exhaled NO levels, but only in never-smoking persons. The estimated alveolar NO increased after ingestion of nitrate in individuals with high nitrate turnover in the pharyngo-oral tract. Pulmonary arterial hypertension, but not other forms of PH, was associated with decreased bronchial NO flux, whereas PH of all etiologies was related to increased alveolar NO concentrations.

Conclusion: Smoking history and IgE sensitization, that are known determinants of exhaled NO, affected the bronchial and alveolar contributions to exhaled NO differently. The limitations of the simple NO pulmonary exchange models were highlighted by the paradoxical effects on estimated alveolar NO when modulating the NO production proximally, in the pharyngo-oral tract. Predominance of non-eosinophilic inflammation in ever-smoking patients with asthma could explain the poor association between the presence of asthma and exhaled NO in these patients. Different pathophysiological changes in terms of bronchial NO production and exchange were related to the etiology of pulmonary hypertension.

Keywords: exhaled nitric oxide, alveolar NO, bronchial NO flux, extended NO analysis, asthma, smoking, snus, IgE-sensitization, pharyngo-oral tract, nitrate, nitrite, pulmonary hypertension

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This PhD thesis is based on the following original papers, referred to in the text by their Roman numerals:

I. A Malinovschi, C Janson, T Holmkvist, D Norbäck, P Meriläinen and M Högman. Effect of smoking on exhaled nitric oxide and flow-independent nitric oxide exchange parameters *Eur Respir J. 2006; 28: 339-345*

II. A Malinovschi, C Janson, T Holmkvist, D Norbäck, P Meriläinen and M Högman. IgE sensitisation in relation to flow-independent nitric oxide exchange parameters *Respir Res. 2006; 7:92*

III. A Malinovschi, C Janson, M Högman, G Rolla, K Torén, D Norbäck and A-C Olin. Both allergic and nonallergic asthma are associated with increased $FE_{\text{NO}}$ levels, but only in never-smokers *Allergy 2008 (in press)*

IV. A Malinovschi, C Janson, L Holm, L Nordvall and K Alving. Basal and induced NO formation in the pharyngo-oral tract influences estimates of alveolar NO levels *(Submitted)*

V. A Malinovschi*, D Henrohn*, A Eriksson, JO Lundberg, K Alving, G Wikström. Increased plasma and salivary nitrite and decreased bronchial contribution to exhaled NO in pulmonary arterial hypertension *(Submitted)*

*These authors contributed equally to the present work

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADMA</td>
<td>Asymmetric Dimethylarginine</td>
</tr>
<tr>
<td>ATS</td>
<td>American Thoracic Society</td>
</tr>
<tr>
<td>BHR</td>
<td>Bronchial Hyperresponsiveness</td>
</tr>
<tr>
<td>Calv(_{NO})</td>
<td>Alveolar Concentration of Nitric Oxide</td>
</tr>
<tr>
<td>Caw(_{NO})</td>
<td>Airway Concentration of Nitric Oxide</td>
</tr>
<tr>
<td>CHX</td>
<td>Chlorhexidine</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic Obstructive Pulmonary Disease</td>
</tr>
<tr>
<td>Daw(_{NO})</td>
<td>Airway Transfer Factor for Nitric Oxide</td>
</tr>
<tr>
<td>EBC</td>
<td>Exhaled Breath Condensate</td>
</tr>
<tr>
<td>ECRHS</td>
<td>European Community Respiratory Health Survey</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial Nitric Oxide Synthase</td>
</tr>
<tr>
<td>ERS</td>
<td>European Respiratory Society</td>
</tr>
<tr>
<td>FE(_{NO})</td>
<td>Fraction of Exhaled Nitric Oxide</td>
</tr>
<tr>
<td>FEV(_1)</td>
<td>Forced Expiratory Volume in 1 second</td>
</tr>
<tr>
<td>IgE</td>
<td>Immunoglobulin E</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible Nitric Oxide Synthase</td>
</tr>
<tr>
<td>J'aw(_{NO})</td>
<td>Bronchial NO Flux</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal Nitric Oxide Synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric Oxide Synthase</td>
</tr>
<tr>
<td>NYHA</td>
<td>New York Heart Association</td>
</tr>
<tr>
<td>PAH</td>
<td>Pulmonary Arterial Hypertension</td>
</tr>
<tr>
<td>PH</td>
<td>Pulmonary Hypertension</td>
</tr>
<tr>
<td>ppb</td>
<td>parts per billion</td>
</tr>
<tr>
<td>TMAD</td>
<td>Trumpet Model and Axial Diffusion</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
Introduction

Airway inflammation

Inflammometry concept

The concept of inflammometry in airway disease was first introduced by de Jongste et al.\(^1\) to emphasize that it is necessary to quantify the degree of inflammation in the airways in order to rationally treat an inflammatory disease (such as asthma).

An objective assessment of inflammation would theoretically lead to better targeting of anti-inflammatory treatment as well as better titration of anti-inflammatory medication. Moreover, markers of inflammation could lead to an early diagnosis of subclinical disease, predict exacerbations and help in making differential diagnosis.

Inflammometry - evidence

Airway inflammation is an important component of both asthma and chronic obstructive pulmonary disease (COPD). There is evidence that inflammation is a dimension of these respiratory diseases that correlates poorly with other dimensions of these diseases, such as lung function, bronchial hyperresponsiveness (BHR) and symptoms\(^2,3\), indicating that the value of the latter measurements as surrogate markers of inflammation is limited.

Consistent evidence exists indicating that eosinophilic inflammation is the most reliable predictor of a response to corticosteroid treatment in patients with asthma\(^4,5\). Moreover, this finding can be extended to patients with airway disease in that this is valid for both eosinophilic bronchitis\(^6\) and COPD\(^7\), as recently pointed out by Pavord et al.\(^8\).

The surrogate markers used to date to assess airways eosinophilic inflammation in studies aiming to titrate the inhaled corticosteroids in asthmatic patients according to the degree of inflammation have been sputum eosinophils\(^9\), serum eosinophil cationic protein (ECP)\(^10\) and exhaled nitric oxide\(^11\). BHR to methacholine has also been studied in a similar setting\(^12\) even though its use as a surrogate marker of inflammation is controversial\(^13\).
Exhaled nitric oxide

Nitric oxide

There has been an interesting evolution of the small and simple molecule nitric oxide (NO) from a noxious environmental pollutant destroying the ozone layer and causing acid rain to an essential molecule in the physiology of the most animals when it was discovered in the 1980s that NO is the endothelium-derived relaxing factor.

Nitric oxide regulates many body functions, including blood pressure, respiration, blood clotting, bacterial killing, cancer prevention, brain and sexual function. With special regard to the respiratory tract, it is possible to attribute to NO a list of different roles:

a. physiological
   - relaxation of the airway smooth muscle → bronchodilation
   - bronchoprotection
   - involved in the regulation of vascular basal tone and counteraction of hypoxic vasoconstriction in pulmonary circulation
   - stimulatory effect on airway submucosal gland secretion
   - control of ciliary beat frequency of airway epithelial cells

b. pathophysiological
   - airway inflammation
   - airway smooth muscle proliferation

(adapted from Ricciardolo et al.)

NO can be enzymatically produced from the amino acid L-arginine by means of NO synthases (NOS). There are three NOS classically described, although there is some evidence even for a fourth mitochondrial form. The reaction is both oxygen- and NADPH-dependent and yields the coproduct L-citrulline in addition to NO in a 1:1 stoichiometry.

A brief summary of the location of these three isoforms in the airways is given below:

a. endothelial NOS (eNOS) – localized in endothelial cells of pulmonary and bronchial blood vessels, human bronchial epithelium and type II human alveolar epithelial cells.

b. neuronal NOS (nNOS) – localized in airway nerve fibers (especially in postganglionic parasympathetic nerves innervating airway smooth muscle and submucosal glands)
c. inducible NOS (iNOS) – respiratory epithelial cells, macrophages, alveolar type II epithelial cells, lung fibroblasts, airway and vascular smooth muscle cells, mast cells, endothelial cells, neutrophils and chondrocytes.

Additional to the NOS-dependent production of NO, a non-NOS production pathway coexists, consisting of the reduction of nitrite to NO. This mechanism of NO formation has been initially demonstrated in the stomach, but as recently reviewed by Lundberg et al., nitrite can also be reduced to NO in the vasculature, pharyngo-oral tract, on the skin surface, in urine and in the lower gastrointestinal tract.

Exhaled NO – origin and information

The measured exhaled NO is considered to originate preponderantly in the respiratory epithelial cells, and bronchial epithelial iNOS has been shown to be a major determinant of orally exhaled NO levels. Moreover, selective inhibition of iNOS has lead to a reduction of exhaled NO levels with approximately 85% in asthmatic patients and about 75% in healthy individuals.

The pharyngo-oral tract contributes to the measured exhaled NO as studies in tracheostomized patients have demonstrated up to 50% lower levels when breathing through the tracheostomy stoma than during normal oral breathing. This contribution is probably dual: a NOS-dependent production in that NOS expression was found in the oral mucosa and a non-NOS production through acidic and bacterial reduction of nitrite to NO. Reducing the bacterial production of NO with a chlorhexidine mouthwash decreases the levels of exhaled NO by about 20% in healthy individuals and a reduction of similar magnitude of exhaled NO is obtained by spraying a NOS inhibitor in the oral cavity. The production of NO in the pharyngo-oral tract is augmented by pharmacologically or dietary nitrate intake. A recent publication has demonstrated that the increase of exhaled NO persists up to 15 hours after intake of a nitrate-rich meal.

Exhaled NO has been shown to be a good surrogate marker of eosinophilic airway inflammation. Exhaled NO levels correlate well with eosinophil count in induced sputum, eosinophils in bronchial biopsy material and bronchoalveolar lavage fluid in asthmatics. Moreover, FE\textsubscript{NO} levels were reported to relate to blood eosinophils and sputum ECP in patients with asthma.

Regarding clinical use, exhaled NO is a good marker of steroid-sensitive inflammation as exhaled NO predicts the response to inhaled steroids. The change in exhaled NO after the introduction of inhalation corticosteroids,
which can be observed already after one week of treatment \(^49\), is dose-dependent \(^50\) and the exhaled NO levels return to baseline levels two weeks after discontinuation of steroid therapy \(^51\).

Exhaled NO within the frame of inflammometry

The main field of interest regarding exhaled NO measurements is asthma. Since the original report of Alving et al. \(^52\) showing that \(FE_{NO}\) levels are increased in patients with asthma, more than 1000 publications have studied different aspects on exhaled NO in asthma.

The value of exhaled NO in the clinical management of asthma has been tested in a number of studies, where the following fields of use or benefits have been found:
- reducing steroid doses in children \(^53\) and adults \(^11\) without deterioration of asthma control; improving BHR without changes in steroid usage \(^54\);
- predicting relapse after steroid withdrawal in children with asthma \(^55\);
- predicting responders to inhaled steroid treatment \(^48\);
- monitoring compliance with inhaled steroid treatment \(^56\);
- differential diagnosis of asthma, as low levels of exhaled NO should raise questions about primary ciliary dyskinesia \(^57\) or cystic fibrosis \(^58\).

More studies are necessary before advocating clinical routine use of titrating steroid doses based on exhaled NO as several studies \(^59-61\) in addition to the studies reported above find benefits only in secondary outcomes, but not in primary outcomes when using exhaled NO to tailor the dose of inhaled corticosteroids.

Induced sputum and exhaled breath condensate (EBC) are two other non-invasive methods to assess airway inflammation. In the table below, characteristics of the three methods are listed \(^62,63\).

**Table 1. Description of noninvasive methods used to assess airway inflammation.**

<table>
<thead>
<tr>
<th></th>
<th>Exhaled NO</th>
<th>Induced sputum</th>
<th>EBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung sampling</td>
<td>Central and peripheral airways</td>
<td>Central airways</td>
<td>Central and peripheral airways*</td>
</tr>
<tr>
<td>Type of inflammation</td>
<td>Eosinophilic</td>
<td>Both eosinophilic and neutrophilic</td>
<td>Both eosinophilic and neutrophilic*</td>
</tr>
<tr>
<td>Time for patient</td>
<td>5 minutes</td>
<td>30 minutes</td>
<td>10 minutes</td>
</tr>
</tbody>
</table>
EBC is a promising method, but more studies are needed on selected markers in EBC when making the transition from research to clinical use. Both exhaled NO and induced sputum are well validated methods used to a certain extent in the clinical setting, with exhaled NO having the advantages of being an easy to perform method giving instant results and of assessing both central and peripheral airways (when performing measurements at multiple exhalation flow rates). On the other hand, induced sputum has the advantage of conferring information about neutrophilic inflammation.

BHR to indirect stimuli is advocated to reflect airways inflammation to a larger extent than classical stimuli (e.g., methacholine), which act directly on the bronchial smooth muscle. However, this finding appears to be limited to steroid-naïve asthmatics in that no relation between BHR to mannitol or methacholine and airway inflammation was found in well-controlled, steroid-treated asthmatics.

### Peripheral and central contributions to exhaled NO

**Flow-dependency of exhaled NO**

Exhaled NO levels are dependent on the exhalation flow rate ($V_E$), with lower levels of NO at higher exhalation flow rates (Figure 1, closed triangles). The exponential aspect of the plot indicates that exhaled NO must have a dual origin: alveolar, where a steady-state is reached – the alveolar concentration of NO ($Calv_{NO}$), and bronchial, where the contribution to exhaled NO is by diffusion from the airway wall, characterized by airway wall concentration of NO ($Caw_{NO}$) and airway NO transfer factor ($Daw_{NO}$), according to Fick’s diffusion law (Figure 2):

$$FE_{NO} = Caw_{NO} - (Caw_{NO} - Calv_{NO}) \cdot e^{-\frac{Daw_{NO}}{V_E}}$$
Figure 1. Exhaled nitric oxide levels (left Y-axis, closed triangles), and nitric oxide output (right Y-axis, closed diamonds) as a function of exhalation flow rate.

Figure 2. The contribution of airway wall to measured orally exhaled NO as a function of the exhalation flow rate (5, 50 or 500 mL/s) (adapted from Silkoff et al. 68). The simulation is made for $C_{awNO}=100$ ppb, $D_{awNO}=10$mL/s, $CalvNO=2$ ppb.
This observation was the basis for the modelling of NO production and exchange in the airways, as well as, for the first introduced model, i.e. the slope-intercept model. This model uses the linear part of the plot of NO output vs. exhalation flow rates (Figure 1, diamonds) that occurs at high exhalation flow rates, usually higher than 50 mL/s in healthy persons and 100 mL/s in patients with asthma. This linear increase can be described by means of the slope, which corresponds to the alveolar NO concentration (Calv_{NO}) and intercept, which corresponds to the bronchial NO flux ($J'_{aw_{NO}}$):

$$VE_{NO} = J'_{aw_{NO}} + \dot{V}_E \cdot Calv_{NO}$$

The first biological validation of the slope-intercept model was done by Lehtimäki et al. These authors demonstrated higher levels of alveolar NO in patients with allergic alveolitis than in healthy individuals and higher levels of bronchial NO in persons with asthma than in healthy controls. A similar study was performed in patients with asthma and in patients with liver cirrhosis and the initial findings of Lehtimäki et al. were confirmed in the case of asthmatic subjects. The cirrhotic patients behaved like the allergic alveolitis patients, presenting with increased alveolar NO concentrations. Another study performed in patients with mild-to-moderate and severe asthma has demonstrated good correlations between alveolar NO and tests of peripheral lung function in patients with severe asthma.

Several models have been suggested to describe the peripheral (alveolar) and central (bronchial) contribution to exhaled NO levels (see George et al. for a review). The characteristics of some of the models are presented in Table 2.

### Table 2. Summary of the methods used to evaluate peripheral and central contributions to exhaled NO.

<table>
<thead>
<tr>
<th>Method</th>
<th>Flow rates</th>
<th>Daw_{NO} and Caw_{NO}*</th>
<th>Mathematical method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tsoukias</td>
<td>≥ 2</td>
<td>100 – 500 mL/s</td>
<td>No</td>
</tr>
<tr>
<td>Pietropaoli</td>
<td>≥ 2</td>
<td>100 – 500 mL/s</td>
<td>No</td>
</tr>
<tr>
<td>Silkoff</td>
<td>≥ 2</td>
<td>15 - 50 mL/s</td>
<td>Yes</td>
</tr>
<tr>
<td>Högman</td>
<td>3</td>
<td>5, 100, 500 mL/s</td>
<td>Yes</td>
</tr>
</tbody>
</table>

* Ability of the model to estimate Caw_{NO} and Daw_{NO}, both components of bronchial flux, in order to further describe NO production and exchange in the conducting airways.
All the described models are based on the assumption that the lower respiratory tract is built from two compartments, the bronchial compartment that is cylinder-formed and the alveoli. Further, possible improvements of the models include the consideration of the trumpet shape of the airways with adjustments for axial diffusion⁷⁷, the use of a three compartment model ⁷⁸ or correction for ventilation heterogeneity in a multicompartment model ⁷⁹.

However, the increased complexity of the mathematical models does not guarantee enhanced clinical value. Therefore, the vast majority of the published articles using NO flow-independent parameters are based on the slope-intercept model ⁷⁰. Recent studies have proposed easy-to-use mathematical adjustments of this model to correct for the trumpet shape of the airways and axial diffusion⁸⁰,⁸¹.

Determinants of exhaled NO and NO flow-independent exchange parameters

Exhaled NO levels are affected by a series of factors⁸², some of which are method-related while others depend on patient characteristics, both disease- and non-disease-related (Table 3).

Table 3. Factors known to affect exhaled NO levels.

<table>
<thead>
<tr>
<th>Technique-related factors</th>
<th>Non-disease-related patient factors</th>
<th>Disease-related factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral pressure</td>
<td>Age</td>
<td>Respiratory diseases*</td>
</tr>
<tr>
<td>(nasal contamination)</td>
<td>Gender and height</td>
<td>- asthma</td>
</tr>
<tr>
<td>Flow rate</td>
<td>Spirometric maneuvers</td>
<td>- allergic rhinitis</td>
</tr>
<tr>
<td>Breathhold</td>
<td>Airway caliber</td>
<td>Pulmonary hypertension</td>
</tr>
<tr>
<td>Ambient NO</td>
<td>Food and beverages</td>
<td>Systemic diseases*</td>
</tr>
<tr>
<td>Temperature</td>
<td>Exercise</td>
<td>- HIV</td>
</tr>
<tr>
<td>Humidity</td>
<td>Smoking</td>
<td>- systemic sclerosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Infections</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IgE sensitization</td>
</tr>
</tbody>
</table>

* The list is not complete, only some examples are given. Factors in **bold** represent factors mainly investigated in the current thesis.
Several studies have focused on the determinants of exhaled NO in an epidemiological setting. The results from two large population-based studies\textsuperscript{83,84} are summarized in Table 4. It should be noted that the explanatory value of these multivariate models is poor because gender, height and age could explain only 11% of the FENO levels in non-smoking, nonatopic healthy individuals\textsuperscript{85}. Travers et al.\textsuperscript{84} found that the multivariate model including all the variables listed in Table 4 could account only for 22% of the variability of FENO values in a random population sample.

Table 4. Determinants of exhaled NO as reported by two of the largest published epidemiological studies.

<table>
<thead>
<tr>
<th></th>
<th>Olin et al.\textsuperscript{83}</th>
<th>Travers et al.\textsuperscript{84}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male gender</td>
<td>↑*</td>
<td>↑</td>
</tr>
<tr>
<td>Height</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Age</td>
<td>↑</td>
<td>Not significant</td>
</tr>
<tr>
<td>Atopy</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Current smoking</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Previous smoking</td>
<td>Not significant</td>
<td>↓</td>
</tr>
<tr>
<td>Asthma</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Allergic rhinitis</td>
<td>Not determined</td>
<td>↑</td>
</tr>
</tbody>
</table>

* significant in a univariate analysis, but not in a multivariate analysis

By modelling NO exchange and production in the airways, we can gain greater insight into the pathophysiology of inflammatory airway diseases. This can be achieved by describing where the changes in exhaled NO originate along the airway tree. For example, an increase of both bronchial and alveolar contributions to exhaled NO explained the increased levels of FENO observed in patients with severe asthma (Table 5). Moreover, more subtle changes in the periphery of the lung may be identified when normal levels of FENO are recorded, such as in the case of allergic alveolitis or obstructive sleep apnea (Table 5).

Concerning the non-disease-related factors investigated in the present thesis, current knowledge about the impact on exhaled NO and NO flow-independent exchange parameters is summarized in Table 6.
Table 5. Disease-associated changes in exhaled NO and NO flow-independent parameters.

<table>
<thead>
<tr>
<th>Disease</th>
<th>$\text{FE}_{\text{NO}}$</th>
<th>Bronchial compartment</th>
<th>Alveolar Compartment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well-controlled asthma</td>
<td>↑ $^{86,87}$</td>
<td>↑ J’aw$_{\text{NO}}$ $^{86,87}$</td>
<td>↔ Calv$_{\text{NO}}$ $^{86,87}$</td>
</tr>
<tr>
<td>Refractory asthma</td>
<td>↑ $^{88}$</td>
<td>↑ J’aw$_{\text{NO}}$ $^{88}$</td>
<td>↑ Calv$_{\text{NO}}$ $^{88}$</td>
</tr>
<tr>
<td>Severe asthma</td>
<td>↑ $^{89}$</td>
<td>↑ J’aw$_{\text{NO}}$ $^{89}$</td>
<td>↑ Calv$_{\text{NO}}$ $^{75,89}$</td>
</tr>
<tr>
<td>Nocturnal asthma</td>
<td>↑ $^{90}$</td>
<td>↑ J’aw$_{\text{NO}}$ $^{90}$</td>
<td>↑ Calv$_{\text{NO}}$ $^{90}$</td>
</tr>
<tr>
<td>Obstructive sleep apnea</td>
<td>↔ $^{91}$</td>
<td>↔ J’aw$_{\text{NO}}$ $^{91}$</td>
<td>↓Calv$_{\text{NO}}$ $^{91}$</td>
</tr>
<tr>
<td>Allergic alveolitis</td>
<td>↔ $^{71}$</td>
<td>↔ J’aw$_{\text{NO}}$ $^{71}$</td>
<td>↑Calv$_{\text{NO}}$ $^{71}$</td>
</tr>
<tr>
<td>Cystic fibrosis</td>
<td>↔ $^{92,93}$ or ↓ $^{58}$</td>
<td>↔ J’aw$<em>{\text{NO}}$ $^{92,93}$, ↓Caw$</em>{\text{NO}}$ $^{92}$</td>
<td>↑ Calv$<em>{\text{NO}}$ $^{93}$ or ↓Calv$</em>{\text{NO}}$ $^{92}$</td>
</tr>
<tr>
<td>Primary pulmonary hypertension</td>
<td>↓ $^{94}$</td>
<td>↓Caw$_{\text{NO}}$ $^{94}$</td>
<td>↔ Calv$_{\text{NO}}$ $^{94}$</td>
</tr>
<tr>
<td>Primary ciliary dyskinesia</td>
<td>↓ $^{95,96}$</td>
<td>↓Jaw$_{\text{NO}}$ $^{95,96}$</td>
<td>↔Calv$<em>{\text{NO}}$ $^{96}$ or ↓ Calv$</em>{\text{NO}}$ $^{95}$</td>
</tr>
</tbody>
</table>

Identifying and quantifying peripheral inflammation in subgroups of asthmatic patients who respond poorly to regular inhaled corticosteroids (e.g. patients with severe asthma or nocturnal asthma) has a potential clinical use. Introducing systemic medications, such as oral corticosteroids $^{88,97}$ or antileukotrienes $^{98}$, may be an indication for this patient group. An alternative would be to try inhaled corticosteroids with ultrafine particles, medication with anti-inflammatory effects on the peripheral airways $^{99}$.

Table 6. Current knowledge about the effects of non-disease-related factors on the levels of exhaled NO and NO flow-independent exchange parameters.

<table>
<thead>
<tr>
<th>Factor</th>
<th>$\text{FE}_{\text{NO}}$</th>
<th>Bronchial Compartment</th>
<th>Alveolar Compartment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current smoking</td>
<td>↓ $^{87,100}$</td>
<td>↓J’aw$<em>{\text{NO}}$ $^{100}$ and ↓Caw$</em>{\text{NO}}$ $^{87}$</td>
<td>↑Calv$<em>{\text{NO}}$ $^{100}$ or ↔ Calv$</em>{\text{NO}}$ $^{87}$</td>
</tr>
<tr>
<td>Previous smoking</td>
<td>↔ $^{83}$ or ↓ $^{84}$</td>
<td>Not determined</td>
<td>Not determined</td>
</tr>
<tr>
<td>IgE sensitization</td>
<td>↑ $^{83}$ or ↔ $^{101}$</td>
<td>Not determined</td>
<td>Not determined</td>
</tr>
<tr>
<td>Nitrate intake</td>
<td>↑ $^{39}$</td>
<td>Not determined</td>
<td>Not determined</td>
</tr>
<tr>
<td>Antibacterial mouthwash</td>
<td>↓ $^{39}$</td>
<td>Not determined</td>
<td>Not determined</td>
</tr>
</tbody>
</table>
Aims

1. To investigate the effects of tobacco use on exhaled NO and NO flow-independent exchange parameters in a population-based setting.

2. To determine which compartment of the lung (bronchial or alveolar compartment) is responsible for the increased levels of exhaled NO seen in IgE-sensitized persons.

3. To analyze determinants of increased exhaled NO levels in allergic respiratory disease.

4. To analyze the value of exhaled NO as a marker of asthmatic inflammation, with special regard to the smoking history and type of asthma (allergic vs. non-allergic).

5. To evaluate the effects of NO contribution from the pharyngo-oral tract on estimated bronchial and alveolar contributions to exhaled NO.

6. To analyze the association between pulmonary hypertension and exhaled NO and NO flow-independent parameters.
Material and methods

Study populations

Papers I and II

Both studies were based on subjects who participated in the European Community Respiratory Health Survey (ECRHS) II, which is the follow-up of an international multicenter study of asthma and allergy (ECRHS). The design of ECRHS I and II has been published in detail\textsuperscript{102,103}. Each participant was sent a brief questionnaire (Stage 1) and, from those who responded, a random sample was invited to undergo a more detailed clinical examination (Stage 2). A "symptomatic" sample consisting of additional subjects who reported symptoms of waking with shortness of breath, asthma attacks or using asthma medication in Stage 1 was also studied. In ECRHS II, subjects who had participated in Stage 2 of ECRHS I were invited to participate in a follow-up study. Subjects answered a standardized questionnaire administered by trained interviewers and underwent lung function tests and blood tests.

In the Uppsala center, 679 subjects were reinvestigated in ECRHS II and of those subjects 288 performed NO measurements at multiple exhalation flow rates (see the flow-chart below in Figure 3 for details). Paper I included only subjects from the “random sample” (n=221) while in Paper II both subjects from the “random” and ”symptomatic” samples were included (n=288).
Figure 3. Flow-chart of selection of subjects for Uppsala center in Paper I and II.

Paper III

This multicenter study was also based on the ECHRS II and included data from three of the 29 participating centers: Uppsala and Gothenburg in Sweden and Turin in Italy where 1548 subjects from the random sample and 496 from the symptomatic sample participated in stage 2 of ECRHS I. Of these subjects, 1484 were included in the ECRHS II. Criteria for inclusion in the present study were to have answered the main questionnaire, to perform FE\textsubscript{NO} and specific IgE measurements. The number of subjects who corresponded to these inclusion criteria was 728, of which 102 were from Turin, 341 from Gothenburg and 285 from Uppsala. Thirty-three subjects on daily inhaled corticosteroids or oral antileukotrienes were excluded leading to a total of 695 subjects analyzed.
Paper IV
Fifteen healthy, non-smoking, non-allergic subjects (nine women and six men) with a median age of 33 years (range 20-47 years) were included in the study.

Paper V
Twenty-four patients (7 males/17 females) with a median age of 60.5 years (range 31-77 years) and a diagnosis of pulmonary hypertension (PH) were included. One patient had physician-diagnosed asthma and another patient was a current smoker, and both were therefore subsequently excluded from the study. The patients were subdivided into two groups with regard to the aetiology of their PH, according to the PH WHO clinical classification system (Venice update): pulmonary arterial hypertension (PAH), corresponding to PH WHO Gr I, (n=13) and PH WHO Gr II-IV (n=9). All the subjects with PH were recruited through the Regional Center for Pulmonary Hypertension at Uppsala University Hospital. In practical terms, patients hospitalized at the Cardiology Department at Uppsala University Hospital qualifying for the study and patients living close to Uppsala (within a radius of 200 km) were recruited.

Twenty-one non-smoking healthy subjects with a median age of 61 years (range 30-78 years) were included as controls. Exclusion criteria were infections in the upper- or lower respiratory tract during the two weeks preceding the study or self-reported physician-diagnosed asthma.

Exhaled NO measurements
The measurements of exhaled NO were performed according to the ATS/ERS guidelines, with the exception of the use of several flow rates in order to calculate the NO flow-independent exchange parameters.

All subjects were instructed to avoid physical exercise and nitrate-rich food the evening before and on the morning of the measurement day. No intake of food and/or beverage was allowed during the hour preceding the measurements. All measurements were performed between 8 and 12 am.

In Papers I and II measurements of exhaled NO were performed at exhalation flow rates of 5, 50, 100 and 500 mL/s. The system used for NO measurements was a computer-based single-breath NO system from Nitrograf (Hässelby, Sweden), which used a chemiluminescence analyzer (Sievers NOA 280; Sievers, Boulder, CO, USA).
In Paper III, different methods, exhalation flow rates and devices were used in the three different study centers: online methods and a flow rate of 50 mL/s in Uppsala and Gothenburg and an offline method and a flow rate of 350 mL/s in Turin. Different NO analyzers were used in the three centers: Sievers NOA 270B, Sievers, Boulder, CA, USA in Uppsala and Turin and Eco Physics NO Analyzer CLD 700 AL, Dürnten, Switzerland in Gothenburg. The detailed methodology of exhaled NO measurements for each center is described in previously published papers from Turin \textsuperscript{104}, Gothenburg \textsuperscript{40} and Uppsala \textsuperscript{87}.

In Paper IV exhaled NO was measured at the flow rates of 100, 200, 300 and 400 mL/s using Aerocrine Exhaled Breath Analyser (Aerocrine AB, Solna, Sweden), which includes the CLD 77 AM chemiluminescence analyzer from Eco Physics AG (Dürnten, Switzerland) that has been described in a previous publication from our group \textsuperscript{105}. To enable the subjects to achieve the target flow rate, dynamic flow resistors were used (Aerocrine AB, Stockholm, Sweden). The exhalation times varied from 6 to 13 seconds, depending on exhalation flow rate, and exhaled NO was always measured on the last three seconds of the exhalation maneuver.

In Paper V, measurements of exhaled NO were performed in duplicate in a random order at the following exhalation flow rates: 20, 50, 100, 200 and 300 mL/s. If the two values of exhaled NO for a specific flow rate differed with more than 10%, a third FE$_{NO}$ measurement was performed. The device used was Aerocrine NIOX Flex\textsuperscript{®} (Aerocrine AB, Solna, Sweden). The time for exhalation maneuvers varied between 6 and 20 seconds, and all the exhaled NO levels were measured on the last three seconds of the exhalation maneuver, independently of the exhalation length.

**NO flow-independent exchange parameters**

**Papers I and II**

The extended NO analysis used in these papers has been previously described and validated \textsuperscript{87}. Using the FE$_{NO}$ measurements at three different exhalation flow rates (5, 100 and 500 mL/s) and an iterative algorithm, it calculates the three flow-independent NO exchange parameters confined to the two compartments, the conducting airways, which are characterized by the airway tissue nitric oxide concentration (Caw$_{NO}$) as well as airway transfer factor (or diffusing capacity) for nitric oxide (Daw$_{NO}$), and the alveoli, characterized by the alveolar nitric oxide concentration (Calv$_{NO}$).
A global airway compartment description was given by the NO bronchial flux - $J'_{awNO}$, which was calculated as $Daw_{NO}(Caw_{NO} - Calv_{NO})$.

Papers IV and V
In order to calculate the contributions from peripheral and central airways to exhaled NO, the slope-intercept model was used\(^6\). The measurements of exhaled NO at flow rates $\geq 100$ mL/s were used to estimate alveolar NO concentration ($Calv_{NO}$) and the bronchial NO flux ($J'_{awNO}$).

Nitric oxide output was calculated by multiplying the $FE_{NO}$ measured at a specific flow rate with the corresponding exhalation flow rate, for example:

$$VE_{NO \ 0.1 \ (pL/s)} = FE_{NO \ 0.1 \ (ppb)} \times 0.1 \ L/s$$

$VE_{NO}$ was converted into nL/min through multiplication by 0.06.

IgE sensitization
In Papers I-III, blood samples were collected for the measurement of total and specific serum immunoglobulin E (IgE) using the Pharmacia CAP System (Pharmacia Diagnostics, Uppsala, Sweden). Specific IgE directed against Derma\textit{tophagoides pteronyssinus}, cat, timothy grass and \textit{Cladosporium herbarum} was measured. The detection of a specific IgE titre $\geq 0.35$ kU/L was used as the definition of sensitization to a specific allergen.

IgE sensitization was defined as sensitization to at least one of the investigated allergens.

Degree of IgE sensitization was defined either as the number of allergens a subject was sensitized to, leading to following categories: nonsensitized, mono- and polysensitized subjects, or as the sum of specific IgE titres against the tested allergens.

Lung function
Forced expiratory volume in 1 s (FEV\textsubscript{1}) was measured with a standardized method with Spiro Medics Spirometer 2130 (Sensor Medics, Anaheim, USA) in Uppsala in papers I-III. The same device was used in Gothenburg while Biomedin Spirometer (Biomedin, Padova, Italy) was used in Turin center in paper III. FEV\textsubscript{1} was expressed as % of the predicted value\(^{106}\).
Questionnaires

In Papers I–III the ECRHS main questionnaire 107 was used to retrieve information about asthma and rhinitis diagnosis, tobacco use and use of anti-inflammatory medication.

Asthma

A person was recorded as having asthma if he/she had ever been diagnosed with asthma and had an asthma attack or one of the following symptoms during the last 12 months: wheezing or whistling in the chest, nocturnal chest tightness or attack of shortness of breath 108.

Further classification into allergic and non-allergic asthma was based on the asthma and IgE sensitization status 108.

Rhinitis

Rhinitis was defined as a positive answer to the question “Do you have any nasal allergy, including hay fever?”.

Tobacco use

For those who answered “yes” to the lead question (“Have you ever smoked for as long as a year?”), additional questions were asked about age on starting, amount smoked currently, whether they had stopped or cut down, and the amount smoked previously. Based on this information, the subjects were classified as never-, ex- or current smokers.

The mean number of cigarettes smoked per day was used to quantify exposure in current smokers. Lifetime exposure to smoking was calculated in pack-years. Latency was defined as the period of time (in years) since ex-smokers had stopped smoking.

Never- and ex-smokers who answered in the affirmative to the question “Have you regularly (most days or nights) been exposed to tobacco smoke in the last 12 months?” were classified as passive smokers.

Oral moist snuff (snus) consumption was registered as a yes/no answer, without recording information about the amount consumed.
Nitrite and nitrate measurements

**Paper IV**

Within the framework of the nitrate load experiment, saliva was collected after each series of measurements (baseline, 60 min after nitrate load, 5 min after CHX mouthwash). The saliva production was unstimulated and 2 mL of saliva was collected each time. The saliva samples were immediately frozen at -80°C for later analysis of nitrite and nitrate levels using a commercially available kit (Nitrate/Nitrite Fluorometric Assay kit, Cayman Chemical Company, Ann Arbor, MI, USA) with a detection limit of 0.2 μM after centrifugation of samples (800 g for 10 min). All saliva samples were analyzed on the same occasion.

**Paper V**

Saliva was collected both from healthy controls and subjects with PH. The saliva production was unstimulated and 2 mL of saliva was collected each time. The saliva samples were immediately frozen at -80°C for later analysis. Samples of blood (10 mL) were collected in tubes containing EDTA. The blood was centrifuged at 800 g for 12 min. Plasma was immediately frozen at -80°C for later analysis.

Nitrate and nitrite in saliva were measured after methanol precipitation of proteins (1:1 v/v) by using a dedicated HPLC system (ENO-20; EiCom, Kyoto, Japan). The method is based on the separation of nitrate by reverse-phase/ion exchange chromatography, followed by on-line reduction of nitrate to nitrite with cadmium and reduced copper\(^{109}\). Reduced nitrite is then derivatized with the Griess reagent and the level of diazo compounds is measured by a visible detector at 540 nm. The retention time for nitrate was 8.6 minutes. All saliva samples were analyzed on the same occasion.

Plasma nitrite and nitrate were determined by gas-phase chemiluminescence with a nitric oxide analyzer (Eco Physics AL 77, Switzerland) after reductive cleavage and subsequent determination of the NO released into the gas phase. The method and apparatus have been described in detail\(^{110}\).

**Study protocol for study IV**

**Chlorhexidine mouthwash experiment**

Two baseline series of exhaled NO measurements were performed in all subjects at an interval of 30 min. Each series consisted of measurements of exhaled NO in duplicate at 100, 200, 300 and 400 mL/s. A third series of
measurements was performed 5 min after the subjects rinsed their mouth with 25 mL chlorhexidine (CHX) mouthwash solution for 30 sec. The CHX mouthwash solution had a pH of 8 and contained chlorhexidine diacetate 0.2% and menthol 0.01%. The series of measurements was complete in less than 10 min, placing it within the timeframe for stable reduction of FE\textsubscript{NO} seen after CHX mouthwash \textsuperscript{39}.

**Nitrate load experiment**

Baseline measurements of exhaled NO in triplicate at 100, 200, 300 and 400 mL/s were done immediately before the ingestion of nitrate. The nitrate load consisted of sodium nitrate (10 mg/kg body weight) dissolved in water (100 mL) which was ingested all at once. This nitrate load is equivalent to, for example, 140 g lettuce or 200 g spinach \textsuperscript{39}. The measurements were repeated 60 min after the nitrate load. A mouthwash with CHX was performed (as described above) after this series of measurements and 5 min after the CHX mouthwash, a third series of exhaled NO measurements was performed.

**Ethics**

**Paper I, II, IV and V**

The study was approved by the regional ethics committee and all subjects gave informed consent for the utilisation of personal data for the purpose of this study.

**Paper III**

Informed consent was obtained from each subject before inclusion in the study. The protocol was approved by the local Ethics Committees in each center (Turin, Italy and Gothenburg and Uppsala, Sweden).

**Statistics**

Statistical analyses were performed using STATA 8.0 software (Stata Corp., 2001, Texas, USA) in Papers I-IV and GraphPAD Prism, version 4.03 (GraphPAD Inc, CA, USA) in Paper V. A p-value of <0.05 was considered statistically significant.
Papers I-III

Exhaled NO and NO flow-independent exchange parameters were log-transformed and were used as dependent variable in different explanatory models. Effects of current or previous smoking and IgE sensitization were tested in Papers I and II in linear regression models (univariate analyses) or multiple linear regression models (multivariate analyses) where adjustments were made for other confounders of interest (gender, height, age, FEV1 (%pred), and IgE sensitization (Paper I) or smoking history (Paper II).

In Paper III due to the use of different FE\textsubscript{NO} measurement techniques, NO analyzers and exhalation flow rates, percentual increase of FE\textsubscript{NO} was used as measure of effect. Percentual increase of FE\textsubscript{NO} was obtained from linear regression models, in the pooled data from the three study centers, where log-transformed FE\textsubscript{NO} was the outcome variable. The regression coefficient for the predictor variable of interest (e.g. current asthma) was back-transformed, as described by Franklin et al.\textsuperscript{111}, in order to obtain percentual increase of FE\textsubscript{NO} (e.g. asthmatics vs nonasthmatics). The effects were studied in linear or multiple linear regression models, in a similar way as in Papers I and II with further adjustments for study sample and center.

Paper IV

The levels of VE\textsubscript{NO}, NO flow-independent exchange parameters, salivary nitrite, nitrate and pH at different time points were compared pairwise using the Wilcoxon signed-rank test. All correlations were tested with the Spearman’s rank correlation test with the exception of the validity of the slope-intercept model where Pearson correlation test was used.

Paper V

Significance testing was based on Mann-Whitney test for the group comparisons involving healthy controls vs. patients with PH. Fisher’s exact test was used to compare the PH groups for differences in gender, NYHA class and use of specific PH medication. The validity of the slope-intercept model was tested by means of Pearson’s correlation test.
Results

Paper I

Smoking habits and exhaled NO

Both previous and current smoking were associated with a significant decrease of FE\textsubscript{NO} levels when compared to never-smoking individuals (Figure 4) (p=0.02 for ex-smoking and p<0.001 for current smoking). No effects of passive smoking on FE\textsubscript{NO 0.05} were found among non-smokers (Figure 4).

![Figure 4](image)

*Figure 4.* The levels of FE\textsubscript{NO 0.05} (geometric mean (95%CI)) in never-, ex- and current smoking persons (left panel) and individuals not-exposed or exposed to passive smoking (right panel), respectively.

These findings were consistent after adjusting for gender, height, age, lung function and IgE sensitization (Table 7).

<table>
<thead>
<tr>
<th></th>
<th>Unadjusted FE\textsubscript{NO} change</th>
<th>Adjusted* FE\textsubscript{NO} change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ex-smoking</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>((n=75))</td>
<td>-22.2% (-34.8, -7.2%)</td>
<td>-20.1% (-33.2, -4.4%)</td>
</tr>
<tr>
<td><strong>Current smoking</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>((n=35))</td>
<td>-38.6% (-51.2, -22.7%)</td>
<td>-40.5% (-52.4, -25.5)</td>
</tr>
</tbody>
</table>

* for gender, height, age, lung function and IgE sensitization or smoking history

No relation of FE\textsubscript{NO} to the number of daily smoked cigarettes in current smokers or the number of pack-years or time since quitting smoking in ex-smokers was found (all p-values>0.05). Oral moist snuff consumption was
not associated with any FE\textsubscript{NO} change among ex-smokers (17.5 ppb (13.8, 22.3) vs. 17.7 ppb (15.0, 20.7)).

Smoking habits and NO flow-independent exchange parameters

Both current and previous smoking were associated with decreased bronchial NO flux (Table 8). There was also a significant decrease of Caw\textsubscript{NO} associated with current smoking (Table 8).

Table 8. Levels of NO flow-independent parameters (geometric mean (95%CI)) in never-, ex- and current smokers.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Never smokers (n=111)</th>
<th>Ex-smokers (n=75)</th>
<th>Current smokers (n=35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>J'aw\textsubscript{NO} (pL/s)</td>
<td>1153 (1011, 1315)</td>
<td>858 (745, 989)*</td>
<td>688 (529, 893)***</td>
</tr>
<tr>
<td>Caw\textsubscript{NO} (ppb)</td>
<td>126 (114, 140)</td>
<td>110 (97, 124)</td>
<td>79 (63, 99)***</td>
</tr>
<tr>
<td>Daw\textsubscript{NO} (mL/s)</td>
<td>9.28 (8.42, 10.2)</td>
<td>7.95 (6.99, 9.05)</td>
<td>8.87 (7.05, 11.02)</td>
</tr>
<tr>
<td>Calv\textsubscript{NO} (ppb)</td>
<td>1.32 (1.13, 1.54)</td>
<td>1.41 (1.14, 1.73)</td>
<td>0.93 (0.67, 1.28)</td>
</tr>
</tbody>
</table>

* p<0.05 and *** p≤0.001 when compared to never-smokers

All these results were confirmed after adjustments for gender, height, age, IgE sensitization and lung function. In this multivariate analysis, a significant negative association between current smoking and Calv\textsubscript{NO} was found (p=0.004).

No difference in the levels of J’aw\textsubscript{NO}, Caw\textsubscript{NO} or Daw\textsubscript{NO} could be found between non-smoking subjects who were exposed to passive smoking (n=15) and those who were not (n=167). Subjects who were exposed to passive smoking were characterized by higher levels of alveolar NO (2.31 ppb (1.82, 2.93) vs 1.29 ppb (1.13, 1.48), p=0.01). This finding was unchanged after adjustments for confounding variables in a multivariate analysis (p=0.008).

Oral moist snuff and NO flow-independent parameters

The use of oral moist snuff among ex-smokers was not associated with changes in exhaled NO, but it was associated with higher levels of Daw\textsubscript{NO} (9.88 mL/s (8.01, 12.19) vs 7.34 mL/s (6.23, 8.66), p=0.04) and a trend towards lower levels of Caw\textsubscript{NO} (90 ppb (69, 118) vs 116 ppb (102, 134), p=0.06). The levels of Daw\textsubscript{NO} remained significantly higher and the negative association between snuff and Caw\textsubscript{NO} became significant in a multivariate analysis where adjustments were made for gender, age, height, lung function, IgE sensitization, time since quitting smoking and pack-years.
Paper II

IgE sensitization and exhaled NO

IgE-sensitized subjects (n=111) had 44.2% (25.1, 66.3%) higher levels of FENO than non-IgE-sensitized subjects (n=177) (p<0.001). In univariate analyses of individual allergens, cat sensitization was associated with a 57% (35, 84%) FENO increase, timothy with a 43% (21, 69%) FENO increase, mould with a 92% (25, 196%) FENO increase while mite sensitization was related to a non-significant 16% (-10, 50%) FENO increase.

Increased FENO levels were found with allergic mono- and polysensitization (p<0.001 for trend) and the FENO_{0.05} levels increased with the sum of specific IgE titres against tested allergens (r=0.4, p<0.001) (Figure 5).

![Figure 5. FENO_{0.05} in relation to sum of specific IgE titres against tested allergens (both variables log-transformed).](image)

IgE sensitization and NO flow-independent parameters

There was an association between the degree of IgE sensitization and both CawNO and DawNO, either when measured as polysensitization (p=0.02 for trend for CawNO and p<0.001 for trend for DawNO) or sum of specific IgE titres (Table 9).

**Table 9. The magnitude of change in NO flow-independent parameters (log-transformed) per 1 log-transformed kU/L of sum of specific IgE in a univariate and multivariate model.**

<table>
<thead>
<tr>
<th>Association</th>
<th>Unadjusted*</th>
<th>Adjusted**</th>
</tr>
</thead>
<tbody>
<tr>
<td>CawNO – sum specific IgE</td>
<td>0.13 (0.08, 0.19)*</td>
<td>0.12 (0.06, 0.18)*</td>
</tr>
<tr>
<td>DawNO – sum specific IgE</td>
<td>0.13 (0.07, 0.19)*</td>
<td>0.11 (0.05, 0.17)*</td>
</tr>
<tr>
<td>CalvNO – sum specific IgE</td>
<td>0.02 (-0.07, 0.12)</td>
<td>0.03 (-0.06, 0.12)</td>
</tr>
</tbody>
</table>

* p-value<0.001 for the association in the respective model
** for gender, height, age, lung function and smoking history
Determinants of NO flow-independent parameters

In the multivariate model used in Table 9, a positive association was found between $\text{Daw}_{\text{NO}}$ and height ($p=0.006$) as well as between $\text{Calv}_{\text{NO}}$ and age ($p<0.001$). Females were characterized by higher $\text{Calv}_{\text{NO}}$ levels ($p=0.003$).

Explanatory value of models using degree of IgE sensitization

The IgE sensitization’s explanatory value of $\text{FE}_{\text{NO}}$ levels in a linear model was higher when using degree of IgE sensitization (sum of specific IgE) ($R^2=0.16$) than using a qualitative assessment (presence/absence of IgE sensitization) ($R^2=0.08$). Similar differences were found when analyzing the effect of individual allergen sensitizations, expressed as qualitative or quantitative variable, on $\text{FE}_{\text{NO}}$ levels or NO flow-independent parameters.

Presence of respiratory disease and exhaled NO

The presence of respiratory disease (rhinitis or asthma), as well as the degree of IgE sensitization were found to be positively associated with the $\text{FE}_{\text{NO}}$ levels (Table 10).

Different patterns of exhaled NO in relation to presence of respiratory disease were found when stratifying subjects according to the presence of IgE sensitization. In non-IgE sensitized subjects, similar $\text{FE}_{\text{NO}}$ levels (geometric mean (95% CI)) were found in healthy subjects (17.1 ppb (15.7, 18.7)), subjects with rhinitis (18.7 ppb (15.5, 22.7)) and asthmatic subjects with or without rhinitis (20.2 ppb (13.6, 30)). Among IgE-sensitized subjects, $\text{FE}_{\text{NO}}$ levels increased with the presence of respiratory disease (19.3 ppb (15.2, 24.6) in healthy atopics vs 25.5 ppb (20.4, 31.8) for rhinitic and 30.7 ppb (23.7, 39.8) for asthmatic subjects).

The presence of asthma or rhinitis was no longer associated with increased $\text{FE}_{\text{NO}}$ in a multivariate model, after adjusting for the degree of IgE sensitization (Table 10).

| Table 10. The percentual $\text{FE}_{\text{NO}}$ change (mean (95% confidence interval)) associated with presence of asthma, rhinitis or degree of IgE sensitization. |
|----------------------------------------|----------------------------------------|----------------------------------------|
| **Rhinitis**                           | **Unadjusted* $\text{FE}_{\text{NO}}$ change** | **Adjusted§ $\text{FE}_{\text{NO}}$ change** |
| Rhinitis                               | 34.9% (14.8, 54.9%)                     | 12.2% (-4.5, 31.8%)                    |
| Asthma                                 | 51.4% (23.0, 82.0%)                     | 17.5% (-6.7, 44.5%)                    |
| Degree of IgE sensitization            | 51.4% (34.9, 69.8%)                     | 41.3% (20.2, 62.2%)                    |

* All results adjusted for gender, height, age, lung function and smoking history
§ After further adjustments for variables in the table
# Results expressed as increase per 1 log unit sum of specific IgE
Paper III

Determinants of exhaled NO levels in the multicenter study

Current smoking and female gender were associated with lower FE\textsubscript{NO} levels while increased height, presence of atopy and current asthma were all associated with increased FE\textsubscript{NO} levels in the univariate analyses (Table 11).

Table 11. Determinants of exhaled NO in the multicenter study.

<table>
<thead>
<tr>
<th></th>
<th>Unadjusted FE\textsubscript{NO} change</th>
<th>Adjusted\textsuperscript{§} FE\textsubscript{NO} change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female gender</td>
<td>-26.3% (-33.6, -18.2%)</td>
<td>-13.2% (-23.5, -1.5%)</td>
</tr>
<tr>
<td>Age (per 10 yrs)</td>
<td>0% (-7.2, 7.6%)</td>
<td>6.0% (-0.6, 13.1%)</td>
</tr>
<tr>
<td>Height (per 10 cm)</td>
<td>17.7% (11.7, 23.9%)</td>
<td>10.3% (3.4, 17.7%)</td>
</tr>
<tr>
<td>Atopy</td>
<td>41.9% (27.1, 58.3%)</td>
<td>26.6% (14.6, 39.9%)</td>
</tr>
<tr>
<td>Ex-smoking</td>
<td>-8.9% (-18.8, 2.4%)</td>
<td>-9.2% (-18.2, 0.9%)</td>
</tr>
<tr>
<td>Current smoking</td>
<td>-43.2% (-50.2, -35.1%)</td>
<td>-39.7% (-46.6, 32.0%)</td>
</tr>
<tr>
<td>Current asthma</td>
<td>46.7% (26.3, 70.3%)</td>
<td>45.8% (25.6, 69.3%)</td>
</tr>
</tbody>
</table>

\* for all the variables in the table, lung function, study sample and center

Effects of asthma and atopy on exhaled NO

Patients with allergic asthma (n=65), but not patients with non-allergic asthma (n=33) were characterized by significantly higher levels of FE\textsubscript{NO} than nonatopic nonasthmatics (n=432) (Figure 6A). Both patients with nonallergic and allergic asthma had higher levels than nonatopic nonasthmatics (p<0.05 both) after adjustments for gender, height, age, lung function, smoking history, study sample and center. Moreover, the FE\textsubscript{NO} levels in patients with allergic asthma were higher than in patients with non-allergic asthma (p<0.05) (Figure 6B).

![Figure 6. The percentual increase of FE\textsubscript{NO} (mean (95%CI)) in patients with nonallergic or allergic asthma, compared to nonatopic nonasthmatics, before (Panel A) and after (Panel B) adjustments for gender, height, age, lung function, study sample and center.](image-url)
Smoking history effects

Subjects were divided into three strata according to the smoking history: never- (n=318), ex- (n=224) and current smokers (n=146). In a univariate analysis, the atopy-associated increase of $F_{ENO}$ was consistent in all the three strata while the asthma-associated increase was modulated by smoking history in a fashion described in the table below (Table 12).

<table>
<thead>
<tr>
<th></th>
<th>Never smokers</th>
<th>Ex-smokers</th>
<th>Current smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Atopy</strong></td>
<td>41% (21%, 65%)</td>
<td>37% (15%, 64%)</td>
<td>42% (12%, 80%)</td>
</tr>
<tr>
<td><strong>Current asthma</strong></td>
<td>70% (39%, 108%)</td>
<td>46% (14%, 88%)</td>
<td>-12% (-38%, 24%)</td>
</tr>
</tbody>
</table>

After dividing the subjects upon their smoking and atopy status, an asthma-associated increase of $F_{ENO}$ was found only in never-smoking individuals, both in the nonatopic and atopic group (Figure 7).

![Figure 7](image)

**Figure 7.** Percentual increase of $F_{ENO}$ (mean (95% confidence interval)) in asthmatics vs nonasthmatics in different strata of our study population, obtained after dividing the subjects according to atopy and smoking status. All results are adjusted for gender, age, height, lung function, study sample and center.

Never-smoking subjects with non-allergic asthma (n=14) had 112% (54, 190%) higher $F_{ENO}$ levels than non-asthmatic, never-smoking subjects (n=185), while allergic asthmatic subjects (n=38) had 112% (69, 165%) higher $F_{ENO}$ levels than the same reference category. The results were consistent after adjustments for gender, height, age, lung function, study sample and center.
Paper IV

Effects of antibacterial mouthwash on the levels of exhaled NO

The exhaled nitric oxide output was reduced at all investigated flow rates after chlorhexidine (CHX) mouthwash (p<0.05 all), with a trend towards smaller decrease at the lowest exhalation flow rate as the VE NO reductions (median (interquartile range)) were as following: 9.3 % (0.7, 15%) for 100 mL/s, 16% (4.0, 18.6%) for 200 mL/s, 15% (11, 23%) for 300 mL/s, 15% (6.6, 21%) for 400 mL/s.

The CHX mouthwash resulted in a significant reduction of Calv NO (median (range)) from 1.16 ppb (0.77, 1.96) to 0.84 ppb (0.57, 1.48) (p<0.001) (Figure 8). The levels of J’aw NO were also reduced by the chlorhexidine mouthwash (31.3 nL/min (13.2, 70.9) vs 29.9 nL/min (10.8, 59.9)), but this reduction was not significant (p=0.13). These results were consistent after TMAD adjustments (Figure 8), with a reduction of adjusted Calv NO from 0.62 ppb (0, 1.37) to 0.50 ppb (0, 0.85) after mouthwash (p<0.01).

**Figure 8.** Individual changes (before vs after CHX mouthwash) in alveolar NO for all investigated subjects (left panel – Calv NO, right panel – TMAD-adjusted Calv NO).

Effects of nitrate load

Exhaled nitric oxide output was significantly increased at all investigated flow rates 60 min after nitrate load (open triangles) and decreased 5 min after chlorhexidine mouthwash (closed circles) (Figure 9).

The nitrate load resulted in an increase of J’aw NO (57 nL/min (22, 119) vs. 32 nL/min (12, 60), p<0.001), which graphically (Figure 9) corresponds to the increased intercept (intersection of regression line with Y-axis) after nitrate load. The CHX mouthwash reduced both alveolar NO (corresponding graphically to the slope of the regression line) from 1.47 ppb (0.83, 7.20) to 1.25 ppb (0.72, 2.07) (p<0.05) and J’aw NO, corresponding the intercept, from 57.1 nL/min (22.0, 119) to 42.1 nL/min (20.7, 88.0) (p<0.001).
Figure 9. NO output as a function of exhalation flow rate at baseline (open squares), 60 min after nitrate load (open triangles) and 5 min after CHX mouthwash (closed circles). Values presented as mean and SD.

Interindividual differences in nitrite turnover

The nitrate load was associated with a 32-fold (12, 158) (median (range)) increase of salivary nitrate (p=0.0001) and a 10-fold (4, 50) increase of salivary nitrite (p<0.0001). Nitrate levels were not reduced by the CHX mouthwash (p=0.53), but rather increased compared to baseline (p=0.001), while CHX mouthwash reduced the levels of nitrite by a factor of 6 (4, 25) (p<0.0001) to a level slightly higher than at baseline (p=0.03).

When dividing the subjects according to the increase of salivary nitrite after nitrate load, subjects with a large increase of nitrite (>10-fold increase) were characterized by an increase of both CalvNO (Figure 10) and J’awNO 60 min after nitrate load (p=0.01). Both J’awNO and CalvNO were reduced after CHX mouthwash (p<0.05). All results were consistent after TMAD adjustment.

Figure 10. Individual levels of CalvNO in subjects with small (left panel) or large (right panel) increase of salivary nitrite after nitrate load.
Exhaled NO and NO flow independent parameters in patients with pulmonary hypertension (PH WHO Gr. I-IV)

No differences in the FE\textsubscript{NO} levels in the range of 20-300 mL/s were noted between patients with PH WHO Gr. I-IV and healthy controls (Table 13).

Table 13. \textit{FE\textsubscript{NO} values (median (interquartile range)) for patients with PH (WHO gr I-IV) and healthy controls.}

<table>
<thead>
<tr>
<th>FE\textsubscript{NO}</th>
<th>PH WHO Gr. I-IV (n=22)*</th>
<th>Controls (n=21)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>FE\textsubscript{NO20} (ppb)</td>
<td>30.5 (19.5-37.5)</td>
<td>33.7 (23.1-35.7)</td>
</tr>
<tr>
<td>FE\textsubscript{NO50} (ppb)</td>
<td>15.1 (11.3-19.8)</td>
<td>15.8 (12.6-18.1)</td>
</tr>
<tr>
<td>FE\textsubscript{NO100} (ppb)</td>
<td>9.53 (7.29-11.2)</td>
<td>9.45 (6.93-10.5)</td>
</tr>
<tr>
<td>FE\textsubscript{NO200} (ppb)</td>
<td>6.15 (4.90-7.40)</td>
<td>5.48 (4.37-6.17)</td>
</tr>
<tr>
<td>FE\textsubscript{NO300} (ppb)</td>
<td>4.65 (4.20-6.55)</td>
<td>4.68 (3.27-5.13)</td>
</tr>
</tbody>
</table>

*Some subjects could not perform FE\textsubscript{NO} measurements at all exhalation flow rates.

Similar levels of J’aw\textsubscript{NO} were recorded in patients with PH WHO Gr. I-IV (n=18) and healthy controls (n=20): 557 pL/s (404, 884) vs 731 pL/s (580, 818), p=0.25. However, differences were noted regarding alveolar NO levels with higher levels among patients with PH WHO Gr. I-IV than healthy controls: 2.80 ppb (2.13, 4.88) vs. 1.97 ppb (1.22, 2.49), p=0.01.

Exhaled NO and NO flow independent parameters in patients with PAH vs. patients with PH WHO Gr. II-IV

Exhaled NO was higher at flow rates in the range of 20-200 mL/s in patients with PH WHO Gr. II-IV (n=9) than in patients with PAH (n=13) (p<0.05 all) (Figure 11). No differences in FENO levels were noted between patients with PH WHO gr II-IV and healthy controls. A trend (p=0.09) was found for lower FENO levels at 20 mL/s in patients with PAH than healthy controls.

Figure 11. \textit{FE\textsubscript{NO} levels at flow rates in the range of 20-300 mL/s in patients with PAH (open circles), PH WHO Gr. II-IV (closed diamonds) and healthy controls (closed squares). The symbols denote mean values and whiskers SD. * p<0.05}
Patients with PAH were characterized by lower levels of J’awNO than patients with PH WHO gr II-IV and healthy controls (p=0.02 both) (Figure 12A). Patients with PH were characterized by higher CalvNO levels than healthy controls (p=0.03 and p=0.06 for patients with PAH and PH Gr. II-IV, respectively) (Figure 12B).

![Figure 12](image)

*Figure 12.* Bronchial NO flux (Panel A) and alveolar NO concentration (Panel B) in healthy controls, patients with PAH and patients with PH WHO Gr. II-IV, respectively. The boxplot presents data as median (line), IQR (box) and minimum and maximum values (whiskers).

Plasma and salivary nitrate and nitrite

The levels of plasma and salivary nitrate and nitrite were higher in patients with PH WHO Gr. I-IV, when compared to healthy subjects (p<0.05 all).

Patients with PAH had higher plasma and salivary nitrite as well as higher salivary nitrate than healthy controls (Table 14). No differences were found regarding salivary or plasma nitrate and nitrite between subjects with PAH and PH WHO Gr. II-IV (p>0.05 all).

| Table 14. Plasma (median (range)) and salivary (median (IQR)) nitrite and nitrate levels in patients with PAH and healthy controls. |
|-----------------|-----------------|-----------------|----------------|
|                 | PAH (n=13)      | Controls (n=21) | p-value        |
| **Salivary nitrate (μM)** | 44.7 (25.1, 263) | 9.2 (2.8, 23.6) | <0.01          |
| **Salivary nitrite (μM)**   | 110 (39.8, 255)  | 35.8 (0.54, 122) | 0.047          |
| **Plasma nitrate (μM)**     | 22.7 (11.7, 65.7) | 16.3 (9.8, 40.6) | 0.10           |
| **Plasma nitrite (nM)**     | 120 (27, 268)    | 40.6 (13.8, 532) | 0.01           |
Discussion

Effects of smoking on exhaled NO

Current smoking

Reduced $\text{FE}_{\text{NO}}$ levels have been reported in current smokers in the early 1990s $^{112,113}$, this effect was found both after acute and chronic exposure to smoking $^{114}$. The reduction associated with current smoking is consistently found in epidemiological studies $^{83,84}$, where the magnitude of the decrease can be up to 60-70% $^{83,115}$. We reported reductions of approximately 40% in Papers I and III, which is consistent with the results of Travers et al. $^{84}$.

The probable mechanisms underlying the reduced $\text{FE}_{\text{NO}}$ levels are a reduced NO production due to inhibition of bronchial epithelial iNOS $^{116}$ and/or inadequate supply of cofactors necessary for NO production $^{117}$, or an increased breakdown of NO $^{118,119}$.

The extended analysis of NO explained the decrease of $\text{FE}_{\text{NO}}$ by a reduced contribution from the bronchial compartment. This finding is in accordance with several previous studies $^{87,100}$ that were performed in a limited sample of participants. Moreover, we found decreased levels of alveolar NO, a finding similar to that of Delclaux et al. $^{72}$, but in contradiction with two other studies demonstrating either higher $^{100}$ or similar $^{87}$ levels of $\text{CalV}_{\text{NO}}$ in current smokers. Methodological differences in estimating the alveolar NO as well as different statistical methods that were used may explain the contradictory results. The decreased levels of alveolar NO could reflect an increased permeability of the alveolocapillary membrane $^{120}$ or a decreased production of NO by alveolar macrophages $^{121}$.

Previous smoking

Ex-smokers were characterized by lower $\text{FE}_{\text{NO}}$ levels than never-smokers in Paper I. These results are in accord with those of Robbins et al. $^{122}$, who reported an increase in $\text{FE}_{\text{NO}}$ levels at eight weeks after smoking cessation, but still lower levels of mean oral NO than in healthy non-smoking controls.
However the results do not concur with a study in which the levels of FE\textsubscript{NO} normalized at four weeks after smoking cessation\textsuperscript{100}. A confounding factor in the latest cited study might have been that some of the participants who stopped smoking were IgE-sensitized and therefore had higher baseline FE\textsubscript{NO} levels than control individuals, who were all nonallergic.

The decreased levels of bronchial flux of NO reflect the reduction in NO transfer through the apical membrane of the airway epithelial cells. This may be a consequence of the keratinization of epithelial cells, as seen in oral mucosa\textsuperscript{123} and tracheal epithelium\textsuperscript{124}, which would impede NO diffusion towards the airway lumen.

However in paper III no significant effect of previous smoking was found on exhaled NO. There were differences in the effects of ex-smoking on exhaled NO between study centers regarding the NO levels, with decrease in the Turin and Uppsala centers and no effect in Gothenburg. This discrepancy is difficult to understand, but corresponds with the results from two of the largest epidemiological studies on determinants of exhaled NO\textsuperscript{83,84}, where previous smoking was associated with a decrease of FE\textsubscript{NO} in New Zealand, but not in Gothenburg.

Use of oral moist snuff (snus)

The consumption of snus in relation to exhaled NO has not been studied previously. In our material, use of snus was not associated with any changes in FE\textsubscript{NO} levels. However, changes in Daw\textsubscript{NO} and Caw\textsubscript{NO} could be found using the extended NO analysis. The increase in Daw\textsubscript{NO} associated with snus consumption could be explained by an increased production of NO in the pharyngo-oral tract, as secondary to a larger bacterial colonisation\textsuperscript{125} that may reduce nitrate in snus\textsuperscript{126} to nitrite and further to NO. The reduced airway wall concentration of NO might be a consequence of an increased catabolism of NO in the airways by nicotine-induced transformation of NO to peroxynitrite\textsuperscript{127}.

Effects of allergic sensitization on exhaled NO

The mechanism underlying the increased levels of exhaled NO in IgE-sensitized subjects is not fully understood. Atopic, non-asthmatic persons often have a subclinical airway inflammation\textsuperscript{128}. There is an increased epithelial iNOS activity, which is known to be the main determinant of FE\textsubscript{NO} in humans\textsuperscript{31}. This event is due to cytokine release in connection to bronchial inflammation secondary to exposure to allergens\textsuperscript{129} or effects on gene level\textsuperscript{130}.
In our material the increased levels of FE\textsubscript{NO} associated with IgE sensitization were explained by an increase of both components (i.e. Caw\textsubscript{NO} and Daw\textsubscript{NO}) describing the bronchial compartment. The increase of airway wall concentration of NO can be explained by an increased bronchial epithelial iNOS production of NO, secondary to the causes listed in the paragraph above. The increased NO airways transfer factor is probably explained by morphologically changes in the airways epithelium, as epithelial damage \textsuperscript{131,132}, thickened based membrane \textsuperscript{131,132} and subepithelial fibrosis have been reported in atopic individuals \textsuperscript{133}, changes that might increase the diffusion of NO towards the lumen.

### Quantitative or qualitative assessment of allergic sensitization

Some researchers advocate that the information obtained by quantifying the degree of IgE sensitization is more valuable than the information obtained by only using a qualitative variable (presence/absence of IgE sensitization), for example to predict expression of wheeze \textsuperscript{134}, allergic rhinitis \textsuperscript{135} or in order to identify persons with any allergic disease \textsuperscript{136}.

Several recent studies analyzed the relationship between exhaled NO levels and degree of IgE sensitization, where the degree of IgE sensitization was defined as:

- the number of positive skin prick tests \textsuperscript{137-139},
- the type of allergens to which the person had specific IgE titers over the detection limit \textsuperscript{140},
- skin prick test index, which is the sum of the weal diameters for the investigated allergens \textsuperscript{141,142},
- sum of specific IgE titers against allergens of interest \textsuperscript{143}.

Our study reported that the degree of IgE sensitization is related to higher exhaled NO levels, a finding that agrees with the studies cited above. Moreover, the explanatory value of the linear regression models using FENO as outcome and IgE sensitization as the independent variable was better when using a quantitative degree of IgE sensitization.

### Main determinant of FE\textsubscript{NO} increase in allergic respiratory disease

Our results from Paper II are consistent with recent studies \textsuperscript{137,144} supporting the theory that the increase in FE\textsubscript{NO} values reported in allergic respiratory diseases are more likely due to the atopic status (IgE sensitization) than to the respiratory disease per se. In contrast to this belief, an independent effect of both asthma and sensitization to perennial allergens on FE\textsubscript{NO} was found in
a recent Swedish study \(^{145}\). However, in the respective study no adjustment was applied for the degree of IgE sensitization.

In Paper III, however, we found an independent effect of having asthma on the levels of exhaled NO, after stratifying the participants for presence of IgE sensitization. The apparently divergent findings between Paper II and Paper III regarding the effect of asthma in multivariate models could be explained by the more sensitive way used to assess the degree of IgE sensitization in Paper II. On the other hand, the observation that the allergic rhinitis-associated increase of \(\text{FE}_{\text{NO}}\) is mainly explained by the degree of allergic sensitization could be confirmed in a similar multicenter setting as the one in Paper III \(^{146}\).

### Smoking history and different phenotypes of asthma

Our results suggest that asthmatics that are current smokers have a predominant non-eosinophilic inflammation phenotype as exhaled NO, a marker of eosinophilic inflammation \(^{115}\), was of limited value in differentiating individuals with asthma from those without asthma. We acknowledge the limitation of our study in that no direct measures of eosinophilic inflammation were included, but there is evidence in the literature of increased neutrophilic inflammation and lower eosinophil number in induced sputum of smokers with asthma \(^{147,148}\).

Because similar values of exhaled NO were found in ex-smoking asthmatics and non-asthmatics in Paper III, it may be conjectured that there is a persistence of the neutrophilic phenotype of inflammation in asthmatic ex-smokers. Little evidence exists regarding a possible shift of inflammatory phenotype in asthmatic patients after quitting smoking. Studies of asthma with a short-term follow-up reported lower number of neutrophils in induced sputum both at eight weeks and one year after termination of smoking \(^{149,150}\), but no decrease in neutrophil mediators \(^{150}\). The persistence of the neutrophilic pattern of inflammation after smoking cessation is consistently reported by studies with long follow-up performed in COPD patients \(^{151}\). No relation between time since quitting smoking and exhaled NO was found in our material, suggesting no shift in the inflammation pattern.

### Determinants of exhaled NO in epidemiological studies

As already discussed, the effects of current smoking are of similar magnitude and consistent with previous research (e.g., Travers \textit{et al.}) (Table 15).
Table 15. *Determinants of exhaled NO in relation to our results (Paper III) and those of Travers et al. All results are obtained from multivariate analysis and expressed as percentage increase of FE\textsubscript{NO} (mean (95% confidence interval)).*

<table>
<thead>
<tr>
<th></th>
<th>Paper III</th>
<th>Travers et al. 84</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male gender</strong></td>
<td>15% (1, 31%)</td>
<td>21% (7, 39%)</td>
</tr>
<tr>
<td><strong>Height (per 10 cm)</strong></td>
<td>10% (3, 18%)</td>
<td>14% (1%, 41%)</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td>Not significant</td>
<td>Not significant</td>
</tr>
<tr>
<td><strong>Atopy</strong></td>
<td>27% (15, 40%)</td>
<td>15% (0, 32%)</td>
</tr>
<tr>
<td><strong>Previous smoking</strong></td>
<td>-9 % (-18, 1%)</td>
<td>-13% (-22, -3%)</td>
</tr>
<tr>
<td><strong>Current smoking</strong></td>
<td>-40% (-47, -32%)</td>
<td>-36% (-45, -25%)</td>
</tr>
<tr>
<td><strong>Asthma</strong></td>
<td>46% (26, 69%)</td>
<td>17% (5, 32%)</td>
</tr>
</tbody>
</table>

Differences in the effect of previous smoking on exhaled NO levels have been observed between our study centers, as well as between different studies in the literature 83,84. The effect of gender is also under debate. For instance, in a multivariate analysis, Olin et al. 83 reported no significant effect of gender on exhaled NO and suggested that the lower levels of exhaled NO in females reported in univariate analyses are due to decreased height and lung volumes. However, this finding is contradicted by the studies of Travers et al. 84 and Taylor et al. 152 in which FE\textsubscript{NO} levels were 20-25% higher in male than in female participants after adjusting for possible confounders. These latter findings are in agreement with the reported gender differences in plasma nitrate levels 153, which give an indication of gender differences in endogenous NO production.

The effect of atopy on exhaled NO has been somewhat questioned in small-sized studies 101,145. However, the large population-based studies 83,84,152,154 are consistently reporting that atopy is associated with increased exhaled NO levels. From several large epidemiological studies, the atopy-associated increase of FE\textsubscript{NO} levels was found to be within a relatively wide range, i.e. 16-60% 83,84,152,154. This large variation can be understood through the prism of different degrees of IgE-sensitization in different study populations and/or different levels of allergen exposure, a measure which was not reported in any of the studies. Moreover, methodological differences existed between the above cited studies regarding the number of tested allergens and the method used (skin prick test or specific IgE) to assess IgE sensitization.
The asthma-associated increase of exhaled NO is well documented in small- and large-sized studies. As in the case of atopy, the magnitude of the effect varies across studies (range, 15-35%). The different magnitudes of the asthma-related increase of FE\textsubscript{NO} among studies can be partly explained by a recent study showing that the asthma-related increase of FE\textsubscript{NO} was dependent on the extent of IgE sensitization. Moreover, it is known that allergen exposure further increases the levels of exhaled NO and, as discussed above, allergen exposure data are lacking in the present studies. A methodological explanation may rely in the fact that different studies used different variables to adjust for in the multivariate models. The concomitant adjustment for allergic rhinitis (such as in the study by Travers et al.) might have resulted in a smaller magnitude of the FE\textsubscript{NO} increase associated with asthma. This is because persons with allergic asthma do have to a large extent allergic rhinitis and allergic rhinitis is associated with increased FE\textsubscript{NO} levels.

NO production in the pharyngo-oral tract

The pharyngo-oral contribution to exhaled NO is dual: NOS-dependent and NOS-independent, involving reduction of nitrite to NO. We have modulated the NOS-independent production of NO in the pharyngo-oral tract in Paper IV by intake of nitrate and/or performing an antibacterial mouthwash.

The estimated alveolar NO concentration was affected by the pharyngo-oral tract production of NO in healthy individuals, with a significant decrease observed after the antibacterial mouthwash. However the magnitude of the change in Calv\textsubscript{NO} was relatively small in absolute values. On the other hand, the ingestion of nitrate, which corresponds to ingestion of 200 grams of spinach, could dramatically increase the estimated alveolar NO in some of the individuals characterized by a high nitrate turnover. This finding might be misinterpreted as a sign of peripheral inflammation in the absence of information regarding dietary intake of nitrate.

Cystic fibrosis is a disease associated with higher levels of nitrite in saliva and uncertain effects on the estimated alveolar NO. NO metabolites are also altered in other respiratory diseases, which is suggested by findings in the EBC of patients with asthma. Therefore, it is of interest to test whether the production of NO by reduction of nitrite in the pharyngo-oral tract could mimic inflammatory processes in the lung periphery, as assessed by means of alveolar NO, in different respiratory diseases.
Exhaled NO in pulmonary hypertension

The limited number of studies using exhaled NO measurements in PH have yielded conflicting results. Although most of these studies report decreased exhaled NO levels, others have reported similar or higher exhaled NO levels in patients with PH. It should be noted that the etiology of PH for the patients included in these studies varied widely, as did the techniques used to measure exhaled NO. Furthermore, it was not always clear whether the patients were on any PH-specific treatment or not.

With reference to the etiology of PH, our study analyzed the levels of exhaled NO and NO flow-independent exchange parameters in patients with PH. The majority of the patients included in the study were on specific treatment for PH. Different pathophysiological mechanisms, in terms of NO production and exchange in the airways, appear to underlie pulmonary arterial hypertension (PAH) and secondary forms of PH (PH WHO Gr. II-IV). The lower levels of bronchial NO flux in the presence of higher salivary and plasma nitrite levels in patients with PAH suggest that there is a markedly reduced NOS-derived NO production and/or exchange in the bronchial compartment.

One possible explanation for the lower bronchial production might be a reduced NOS activity as the levels of asymmetric dimethylarginine (ADMA), a potent endogenous inhibitor of NOS, have been reported to be increased in patients with PAH. Another explanation might be the decreased bronchial mucosal area for NO exchange, as secondary to the bronchoconstriction often observed in PAH.

Patients with PH were characterized by increased alveolar NO concentrations. This finding was not related to a specific PH etiology and it probably reflects the dysfunctional alveolocapillary membrane. Further studies are warranted to investigate the importance of this finding as increased alveolar NO might indirectly reflect the ventilation heterogeneity or an increased production of NO in the pharyngo-oral tract by nitrite reduction.

Determinants of NO flow-independent parameters

In contrast to J’aw\textsubscript{NO} or FE\textsubscript{NO}, the alveolar NO concentration was not affected by past smoking and IgE sensitization. The reduction of Calv\textsubscript{NO} associated with current smoking was of small magnitude, being around 0.4 ppb in Paper I. Its relevance should be further investigated. A reduction of alveolar NO of similar magnitude was associated with the male gender. A positive association between age and alveolar NO was found in Paper II, which is a
finding in line with the report of Högman et al. who analyzed the non-smoking healthy subgroup of our population study. Paraskakis et al. reported an increase in the estimated alveolar NO levels with height in children, but no similar results were reported in adults. No association between age and CalvNO was found in our material.

The effects of the above reported patient characteristics on alveolar NO are of low magnitude in comparison with the disease-related increases described in the literature that appear to be in the ppb range. For example, allergic alveolitis and refractory asthma were related to an increase of alveolar NO of about 3 ppb. Certain caution should be exercised when interpreting absolute CalvNO from studies that used different methods to estimate alveolar NO. This point is further discussed in the next section (“In search of the ultimate alveolar NO”).

There was a large increase in estimated alveolar NO after a nitrate load in some individuals. Such an increase might be misinterpreted when alveolar NO is used in identifying peripheral inflammation. Moreover, the relevance of the effects of higher salivary nitrite levels in pathology on estimated alveolar NO should be investigated.

Bronchial NO flux is closely related to FE NO when measured at 50 ml/s. We found that the factors affecting FE NO had similar effects on J’awNO. Consequently, past and current smoking were associated with decreased J’awNO whereas IgE sensitization and nitrate intake were associated with increased levels of J’awNO (Table 16). However, certain discrepancies have been reported between FE NO and bronchial flux. Normal FE NO levels and decreased bronchial NO flux were reported in scleroderma lung disease. Such findings were also observed in our material: patients with PAH were characterized by significantly lower bronchial flux, but not by FE NO when measured at 50 mL/s. Furthermore, an antibacterial mouthwash reduced FE NO levels in healthy individuals, but not the bronchial flux of NO.
In search of the “ultimate” alveolar NO concentration

The real alveolar NO concentration cannot be measured because it would be necessary to perform an exhaled NO measurement at an infinitely high exhalation flow rate. In its absence we use an estimate, Calv_{NO}, which is obtained by means of simple models of pulmonary NO production and exchange. Quite a large spread of Calv_{NO} has been reported in healthy individuals, with mean levels between 1 and 5 ppb \(^{71,72}\). This observation is probably best explained by the choice of exhalation flow rates \(^{172}\). In the absence of a real alveolar NO value it may be argued that it is sufficient to validate the estimates of alveolar NO against studies that used similar flow rates. However, the linearity of the “slope” for the chosen range of flow rates may differ between the pathology of interest and healthy persons \(^{70}\) and therefore a measure of the goodness of fit for the slope-intercept model might be needed to insure accuracy of results.

The importance of axial diffusion of NO from the airways back into the alveoli, which increases the estimates of Calv_{NO}, has been discussed \(^{77}\). Adjustment for axial diffusion appears to be of relevance as patients with stable asthma were characterized by increased levels of alveolar NO that did not persist after adjustments for axial diffusion \(^{81}\). A simplified method of correction for axial diffusion \(^{80}\), used together with the slope-intercept model, proved easy to use in our study.

Our study has highlighted the effect of dietary nitrate intake on the estimated alveolar NO. Individuals with high nitrate turnover in the oral cavity were characterized by an increase of estimated alveolar NO after nitrate intake that could

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### Table 16. Summary of the effects of the factors investigated in the present thesis (Papers I, II, IV) on exhaled NO and NO flow-independent exchange parameters.

<table>
<thead>
<tr>
<th></th>
<th>( \text{FE}_{\text{NO}} )</th>
<th>( J^*_{\text{aw}, \text{NO}} )</th>
<th>( \text{Calv}_{\text{NO}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Current smoking</strong></td>
<td>( \downarrow )</td>
<td>( \downarrow )</td>
<td>( \downarrow )</td>
</tr>
<tr>
<td><strong>Past smoking</strong></td>
<td>( \downarrow )</td>
<td>( \downarrow )</td>
<td>( \leftrightarrow )</td>
</tr>
<tr>
<td><strong>IgE sensitization</strong></td>
<td>( \uparrow )</td>
<td>( \uparrow )</td>
<td>( \leftrightarrow )</td>
</tr>
<tr>
<td><strong>CHX mouthwash</strong></td>
<td>( \downarrow )</td>
<td>( \leftrightarrow )</td>
<td>( \downarrow )</td>
</tr>
<tr>
<td><strong>Nitrate intake</strong></td>
<td>( \uparrow )</td>
<td>( \uparrow )</td>
<td>( \uparrow^{*} )</td>
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* in a subset of participants characterized by high nitrate turnover in the pharyngo-oral tract
be reversed by a chlorhexidine mouthwash. Therefore, if nitrate-rich food was not avoided previous to the study, it might be necessary to perform an antibacterial mouthwash to insure a more accurate estimate of alveolar NO.

It should be stressed that alveolar NO offers valuable information about the peripheral lung, although the points listed above should be kept in mind as well as patient characteristics known to affect CalvNO. Increased alveolar NO is related to peripheral lung function in patients with severe asthma 73, concomitant allergic rhinitis and asthma 173 or nasal polyposis 174. Moreover, alveolar NO is increased in nocturnal asthma 90, which, in turn, is characterized by inflammation of peripheral airways 175. Because alveolar NO levels were negatively related to the diffusing capacity of the lung for carbon monoxide in patients with scleroderma 170,176, alveolar NO appears to give information about the dysfunctionality of the alveolocapillary membrane.

Further characterization of the bronchial compartment

By performing the extended NO measurements, it is possible to further characterize the bronchial contribution to exhaled NO. In the present material we could identify, for example, that a lower concentration of NO in the airways wall explained the decrease of FE_{NO} and J’aw_{NO} in current smokers.

The extended NO analysis is a validated method that could be performed successfully in a population-based material. However, to obtain accurate estimates of the airway wall concentration of NO the method requires the use of a very low exhalation flow rate. This maneuver can be difficult to perform by patients with severe respiratory disease because of the long exhalation times needed to achieve a plateau of exhaled NO 177.

Further characterizing the bronchial compartment to better understand pathophysiological changes of pulmonary NO production and exchange is an undoubted benefit. However, we acknowledge the need for further evidence to support the clinical use of the extended NO analysis in patients with respiratory disease. For example, DawNO has been advocated to reflect a spread of airways inflammation from the central compartment to the periphery 105. When comparing tracheal vs oral exhalation, the relation of DawNO to the mucosal area producing NO is supported by the report of lower DawNO after exclusion of the pharyngo-oral tract 178. Along the same line, estimated CawNO, but not DawNO, was decreased by inhaled steroids in one study 68. However, information on the spread of inflammation towards lung periphery obtained from DawNO should be compared with the one obtained by CalvNO.
Conclusions

1. Both previous and current smoking were associated with decreased exhaled NO levels and its bronchial contribution in a population setting. Moreover, lower alveolar NO levels were found in current smokers.

2. Presence and degree of IgE sensitization were associated with higher exhaled NO levels. This finding was explained by increased airway wall concentration of NO and by increased NO airway transfer factor.

3. Presence of respiratory disease and the degree of allergic sensitization are determinants of exhaled NO levels in patients with allergic respiratory disease. However, presence of asthma and rhinitis was no longer significantly associated with increased exhaled NO levels after adjusting for degree of IgE sensitization.

4. Allergic and non-allergic asthma were associated with increased exhaled NO levels, but only among never-smokers. The limited value of exhaled NO in ever-smoking asthmatics suggests differences in the airway inflammatory profile related to smoking.

5. The NO contribution from the pharyngo-oral tract is reflected in the estimates of alveolar NO, both at baseline conditions and after intake of nitrate. The increase of alveolar NO observed in a subgroup of persons after administration of nitrate could be misinterpreted as a sign of peripheral inflammation.

6. Pulmonary arterial hypertension, but not other forms of pulmonary hypertension, was characterized by lower bronchial contribution to exhaled NO, suggesting altered NO production and/or exchange in the bronchial compartment. Increased alveolar NO levels were found in all patients with pulmonary hypertension, which may reflect a dysfunctional alveolocapillary membrane.
Tracks to follow

The limited value of exhaled NO as an asthma marker in ever-smokers warrants further studies in which asthmatic patients are thoroughly characterized from the inflammatory point of view. Moreover, longitudinal studies on changes in the inflammatory profile of asthmatic patients after smoking cessation would help to close a knowledge gap in the current literature.

Exhaled NO measured at a single flow rate (50 mL/s), according to guidelines, offers very limited information about peripheral processes in the lung. The clinical value of alveolar NO as a surrogate marker of peripheral inflammation in managing asthma remains to be investigated. Therefore, new studies are warranted to analyze the value of alveolar NO in guiding the choice of anti-inflammatory medication targeted at peripheral airways in asthma phenotypes with distal inflammation.

We demonstrated that estimates of alveolar NO levels are increased in individuals with a large salivary nitrite increase after a nitrate intake, making this finding relevant in the context of an unknown dietary intake of nitrate. The relevance of the pharyngo-oral tract contribution to estimated alveolar NO should be investigated in diseases where altered levels of alveolar NO and salivary nitrite levels have been reported (such as cystic fibrosis).

The findings in pulmonary hypertension suggest different pathophysiological mechanisms in terms of NO production and exchange in the airways of patients with pulmonary arterial hypertension as compared with patients with other etiologies of pulmonary hypertension. NO flow-independent exchange parameters may be of value in analyzing the effect of a specific therapy against PH or in identifying the responders to a specific therapy. The value of the bronchial NO flux in differential diagnosis in cases with uncertain etiology of PH remains to be studied.

Concomitant measurements of plasma nitrate and nitrite offer information about the systemic endogenous production of NO while salivary nitrite offers information about the possible non-enzymatic sources to exhaled NO. Corroboration of these measures with exhaled NO and NO flow-independent parameters would provide a better understanding of the NO and NO metabolites “puzzle” in respiratory diseases.
The quantitative description of IgE sensitization appears to be a more reliable marker than absence/presence of IgE sensitization. However, further studies are needed to determine whether an improved measure of the degree of IgE sensitization, as, for example, obtained by extensive characterizations of the allergic profile by microarray techniques, would add even more valuable information.
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