Determinants of fractional exhaled nitric oxide in healthy men and women from the European Community Respiratory Health Survey III

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Abstract

Introduction: The fractional exhaled nitric oxide (F_ENO) is a marker for type 2 inflammation used in diagnostics and management of asthma. In order to use F_ENO as a reliable biomarker, it is important to investigate factors that influence F_ENO in healthy individuals. Men have higher levels of F_ENO than women, but it is unclear whether determinants of F_ENO differ by sex.

Objective: To identify determinants of F_ENO in men and women without lung diseases.

Method: F_ENO was validly measured in 3,881 healthy subjects that had answered the main questionnaire of the European Community Respiratory Health Survey III without airways or lung disease

Results: Exhaled NO levels were 21.3% higher in men compared with women $p<0.001$. Being in the upper age quartile (60.3–67.6 years) men had 19.2 ppb (95% CI: 18.3, 20.2) higher F_ENO than subjects in the lowest age quartile (39.7–48.3 years) $p=0.02$. Women in the two highest age quartiles (54.6–60.2 and 60.3–67.6 years) had 15.4 ppb (14.7, 16.2),

p=0.03 and 16.4 ppb (15.6, 17.1), p=<0.001 higher FENO, compared with the lowest age quartile.

Height was related to 8% higher FENO level in men (p<0.001) and 5% higher FENO levels in women (p=0.008). Men who smoked had 37% lower FENO levels and women had 30% lower levels compared with never-smokers (p<0.001 for both). Men and women sensitized to both grass and perennial allergens had higher FENO levels compared with non-sensitized subjects 26% and 29%, p<0.001 for both.

Conclusion & Clinical Relevance: FENO levels were higher in men than women. Similar effects of current smoking, height, and IgE sensitization were found in both sexes. FENO started increasing at lower age in women than in men, suggesting that interpretation of FENO levels in adults aged over 50 years should take into account age and sex.

Keywords: FENO, healthy population, IgE sensitization, smoking

Introduction

Nitric oxide (NO) serves many functions throughout the body. It is produced by the epithelium as part of the immune defence against pathogens, and is involved in neurotransmission in the peripheral and central nervous systems, as well as the regulation of vascular and bronchiolar tone.

Exhaled NO reflects mainly the respiratory epithelium production of NO, resulting from activation of inducible NO synthase (iNOS), which is controlled by signal transducer and activator of transcription (STAT)-1 under the influence of homeostatic interferon-γ(1). The concentrations are generally low in healthy individuals. However, high concentrations of exhaled NO are seen in chronic inflammatory diseases, such as asthma, mainly due to type 2 inflammation resulting in increased activation of iNOS. Airway infections, especially rhinovirus, and allergic rhinitis are also related to higher levels of exhaled NO(2).

The measurement of fractional exhaled NO (F_ENO) is a useful, non-invasive method to assist with diagnosis of asthma and monitor treatment effects. In recent years, F_ENO has been used as a marker for eosinophilic airway inflammation and asthma(3), and to identify steroid responsiveness in individuals with chronic respiratory symptoms caused by airway inflammation(4).

For F_ENO to be reliable as a biomarker, it is important to know factors that influence F_ENO values. Currently, it is known that F_ENO values are influenced by age(5), gender(5, 6), height(5), atopy(5, 6), smoking(5, 7), respiratory infections(5), environmental factors(8), physical activity(9), and ethnicity(10). Females have consistently been reported to have

lower F_ENO levels than men with about 25% lower levels(5, 6). Some of the explanation might reside in differences in height, another known determinant of F_ENO, but other differences appear to be exist with regard to gender. Moreover, the effect of different known determinants of F_ENO has not been studied with regard to gender. Specifically, the relation with age appears to be different with regard to gender, as a recent publication suggests that after a period in early adulthood with no relation between age and F_ENO, an increase of F_ENO with age is found at age around 45 in women and 59 years in men(11).

In this study, we aimed to describe determinants of F_ENO in men and women, with special emphasis on gender differences, in subjects without lung disease (asthma, chronic obstructive lung function, and emphysema) in the third European Community Respiratory Health Survey (ECRHS III)..

Methods

Study sample

This is a cross-sectional analysis based on the third follow-up of ECRHS (ECRHS III), performed between the years 2010–2013, using data from 25 centres across 11 European countries and Australia.

Briefly, ECRHS is an international multicentre population-based study of asthma and allergy, which was first performed in the early 1990s. The subjects, age 20–44 years, were first randomly selected to complete a short postal questionnaire about asthma symptoms and attacks in the preceding 12 months, current use of asthma medication, and presence of nasal allergies including hay fever. Both a random sample and a symptomatic sample of responders were invited to attend further examinations at their study centre. Current analysis is based on the random population sample. Follow-up studies were performed in 2000–2002 (ECRHS II) and 2010–2013 (ECRHS III). Further details about ECRHS have been published elsewhere(12, 13) and can also be found on the homepage: www.ecrhs.org.

Of 5,483 participants in ECRHS III, 1,004 were excluded due to current asthma and/or asthma symptoms in the last 12 months, 83 due to self-reported physician-diagnosed chronic obstructive pulmonary disease and emphysema, and 176 due to use of inhaled medicines in the last 12 months. Further, 339 subjects with respiratory symptoms at ECRHS I (symptomatic sample)(14) were also excluded. Thus, the final study population included 3,881 participants, aged 39.7–67.6 years (men: 40.0–67.3 years), who underwent F_ENO and other clinical tests, and responded to questions about respiratory symptoms and smoking habits.

Questionnaires and measurements

Participants had to be free from respiratory infections the 2 weeks preceding the clinical examination. An interviewer-led questionnaire contained questions on respiratory symptoms, self-reported asthma, chronic obstructive pulmonary disease and emphysema, use of inhaled drugs in the last 12 months, allergic disorders, and smoking habits. Participants were also asked whether they had any nasal allergies including hay fever.

Current asthma was defined as self-reported asthma with at least one respiratory symptom (wheezing, nocturnal tightness in the chest, attacks of shortness of breath following strenuous activity, at rest or at night-time) in the last 12 months and/or use of asthma medication. Chronic obstructive pulmonary disease (COPD) and emphysema were defined by self-reported physician diagnosis, whereas hay fever was defined by self-report of hay fever or other allergies with similar symptoms in the last 12 months.

Anthropometry

Participant height and weight were measured by trained health technicians and used to calculate body mass index (BMI) (weight [kg]/height [m^2]). BMI was classified in accordance with World Health Organization categories: underweight (< 18.5 kg/m^2), normal weight (18.5–25 kg/m^2), overweight (>25–30 kg/m^2), obese (>30–35 kg/m^2), and very obese (≥ 35 kg/m^2).

Smoking

A smoker was defined as someone who had smoked at least 20 packs of cigarettes or 360 grams of tobacco throughout life, or at least one cigarette a day or one cigar a week for at least one year. Based on smoking habits during the month previous to the study, smokers

were further divided into current and ex-smokers. Never smokers were defined as subjects who never smoked or smoked less than the amount used above to define smokers. Additional questions were asked about age of smoking debut, whether they had stopped or cut down, and the amount currently/previously smoked. The mean number of cigarettes, cigars, cigarillos, and grams of pipe tobacco smoked per day was used to quantify exposure in current smokers(15). Lifetime exposure to smoking was calculated in pack-years (1 pack-year equals smoking 20 cigarettes (1 pack) per day for 1 year). Time since stopped smoking was defined as the period of time (in years) since ex-smokers had quit smoking.

Immunoglobulin E (IgE) sensitization and total IgE

IgE analysis was performed in a single central laboratory (AMC Amsterdam) by using the ImmunoCAP system (Thermo Fisher Scientific, Uppsala, Sweden). In all centres, total IgE and specific IgE were measured against *Dermatophagoides pteronyssinus* (house dust mite), timothy grass, and cat. IgE sensitization was defined as presence of IgE titres for a specific allergen $\geq$ 0.35 kU/L. Group-wise differences were studied regarding F_ENO in different combinations of specific IgE allergens. Group 1: non-sensitized (mite-, cat-, grass-negative); group 2: only sensitized to grass; group 3: only sensitized to perennial allergens (mite- and/or cat-positive); group 4: sensitized to both grass and perennial allergens (mite- and/or cat-positive).

Measurements of exhaled NO

NO measurements were performed in accordance with the recommendations of the American Thoracic Society(16), with the exception that they were performed as single measurements(17). Patients were instructed to avoid smoking, eating or drinking, and strenuous exercise in the hour before the measurement. F_ENO values were measured with

an electrochemical analyser (NIOX MINO; Aerocrine AB, Solna, Sweden) at an expiratory flow rate of 50 ml/s. This device detects exhaled NO values from 5 to 300 ppb. Values below 5 ppb (the lower limit of detection of the device) were recorded in 12 subjects and these received an arbitrary value of 3.5 ppb (5 divided by √2). No values above 300 ppb were recorded in our material.

Statistical methods

All analyses were performed using Stata 14.2 (StataCorp, College Station, TX, USA). The results are described as means, geometric mean values or back-transformed β-coefficient with 95% confidence intervals (CI). Logarithmic transformation was performed for variables with right skewed distribution (F_ENO, total IgE, current cigarettes per day, cigarette packs/10 years, and ex-pack/years).

We have used known determinants of F_ENO from the literature as predictors of eF_ENO in our models: age, gender, height, BMI, smoking, asthma and allergy(18).

All analyses were performed for men and women separately. Bivariate linear regression analyses were used to assess the cross-sectional associations between F_ENO level. Age was divided into age quartiles (39.7–48.3, 48.4–54.5, 54.6–60.2 and 60.3–67.6 years), height, weight, BMI group, smoking history, total IgE level, IgE sensitization to mite, cat, and grass group, and hay fever. Further, bivariate linear regression analysis were performed in relation to the number of cigarettes smoked daily and pack-years, in ex-smokers we analysed F_ENO levels in relation to smoked pack-years and time since stopped smoking. Multiple linear regression analyses were adjusted for age quartile, height, BMI group, smoking habits (three strata: never-, ex-, current smokers), IgE sensitization profile, hay fever, study centre, and self-reported asthma. Interaction analyses between sex and age

group, IgE allergen group (mite, cat and grass), smoking group, current cigarettes, time since stopped smoking, and ex-pack-years were performed on F_ENO as outcome.

These multiple linear regression analyses were also tested for consistency when using a mixed linear model where grouping was done according to study centre(19).

The regression coefficient for the predictor variable of interest (logF_ENO) was back-transformed when the independent variable was normally distributed, by taking the antilog of the estimated transformed F_ENO value. Coefficients should be interpreted as the % change of F_ENO when the independent variables change one unit or in relation to the reference group (for example smokers vs. never-smokers). When both the dependent and independent variables were log-transformed, no reverse transformation was performed (1% increase in the independent variable gave the coefficient percent increase of the dependent variables).

A p value of < 0.05 was considered statistically significant.

Ethics

Informed consent was obtained from all participants prior to inclusion in ECRHS III. Each study centre obtained approval for the study from their regional committee of medical research ethics in accordance with national legislation.

Results

In total, 1,912 (49.3%) of the 3,881 participants were women. The mean age was 54.4 years for men and 53.9 years for women. Exhaled NO levels were higher in men than in women: (geometric mean) 18.2 (95% confidence interval (CI):17.7 to 18.6) vs. 15.0 (14.7 to 15.4) ppb, p<0.001. Baseline characteristics by sex are given in Table 1.

Exhaled NO in relation to anthropometric characteristics.

Men had a 21.3% higher level of F_ENO compared with women. Age was positively associated with F_ENO level. Men in the highest age quartile (60.3–67.6 years) had 19.2 ppb (95% CI: 18.3, 20.2) higher F_ENO than subjects in the lowest age quartile (39.7–48.3 years) p=0.02. Women in the two highest age quartiles (54.6–60.2 and 60.3–67.6 years) had 15.4 ppb (14.7, 16.2), p=0.03 and 16.4 ppb (15.6, 17.1), p=<0.001 higher F_ENO, compared with the lowest age quartile. No crude associations were observed between F_ENO and height, weight, or BMI (Supplementary table 1).

Exhaled NO levels and smoking

Men who never smoked had significantly higher levels of F_ENO (geometric mean [95% CI]) (20.5 ppb (19.8, 21.3)) than ex-smokers (19.3 ppb (18.6, 20.0), p=0.02) and current smokers (12.6 ppb (11.9, 13.3), p<0.001). Women who never smoked had significant higher levels of F_ENO (geometric mean [95% CI]) (16.3 ppb (15.8, 16.8)) than current smokers (10.9 ppb (10.4, 11.5), p<0.001) while no difference between never- and ex-smokers could be found (p=0.19, Figure 2 and Supplemental Table 1). No significant interaction with gender was found on the relation between ex-smoking and F_ENO (p=0.44).

For men that were ex-smokers there was a positive association between F_ENO and the time since they had stopped smoking (coefficient by 10 years: 1.05 (95% CI: 1.02, 1.08)), no significant association was seen in women. Further, no association was found between smoking history (pack-year) and F_ENO level in ex-smoking men and women. Among current smokers, a significant negative association was found between F_ENO level and number of cigarettes per day and pack-years (both p=0.001) in both men and women (Table 2).

Exhaled NO and the relation to IgE sensitization, mite, cat, and grass exposure

Total IgE was associated with higher levels of F_ENO in women (p=0.02), but not in men (p=0.91). Men and women sensitized to mite, cat or grass had higher levels of F_ENO compared with non-sensitized subjects (p<0.001, separate analyses for one allergen at a time).

When studying the allergens in different combinations, men sensitized to grass had 13% (95% CI; 3, 25%) higher F_ENO levels than non-sensitized subjects; for perennial allergens (cat and mite) the levels were 11% (95% CI; 2, 20%) higher, and for grass and perennial allergens 33% (95% CI; 19, 48%) higher. Women sensitized to grass had 11% (95% CI; 2, 22%) higher F_ENO levels than those non-sensitized, for perennial allergens (cat and mite) the levels were 15% (95% CI; 5, 26%) higher, and for grass and perennial allergens 35% (95% CI; 22, 51%) higher (Fig. 3 and Supplemental Table 1).

Multivariate model of determinants of exhaled NO

When we stratified by sex and age groups, the oldest men (60.3–67.6 years) had 9% (95% CI; 1-16%, p=0.02) higher F_ENO values than men in the lowest age quartile (39.7–48.3 years). Women in the two highest age quartiles (54.5–60.2 and 60.3–67.7 years) had 8% (95% CI; 1-14%, p=0.02) and 13% (95% CI; 6-21%, p<0.001) higher F_ENO values, respectively, than those in the lowest age quartile (39.7–48.3 years). Height was related to 8% (95% CI; 5-12%, p<0.001) higher F_ENO level in men and 5% (95% CI; 1-9%, p=0.008) higher F_ENO levels in women (Fig. 4 and Supplemental Table 2). BMI was not significantly related to F_ENO levels (data not shown). Men who smoked had 37% (95% CI; 32-41%) lower F_ENO levels and women 30% (95% CI; 25-34%) lower compared with never-smokers (p<0.001 for both). Among ex-smokers, men had 5% lower F_ENO levels compared with never-smokers (p=0.02). No significant associations were seen between F_ENO and women who were ex-smokers compares with never-smokers.

Men and women sensitized to both grass and perennial allergens had higher F_ENO levels compared with non-sensitized subjects (26% 95% (CI; 14-39%) and 29% (95% CI; 16-43%), p<0.001 for both). Only men showed significant effects of grass sensitization on F_ENO (p=0.02 in men, p=0.13 in women). Women who were sensitized to perennial allergens (mite and/or cat) had higher levels of F_ENO than non-sensitized women (p=0.003). No significant association was seen among men (p=0.09) (Fig. 4 and Supplemental Table 2). No significant association was seen between hay fever and F_ENO levels (data not shown). These results were consistent, both regarding significance and size of the effects, in a mixed linear model with subjects grouped by centre (data not shown).

The only significant interaction between sex and other predictors (age group, IgE allergen group (mite, cat and grass), smoking group, current cigarettes, time since stopped smoking, and ex-pack-years) was between sex and current smoking ($p<0.05$), both in a multiple linear regression model or mixed model (Supplementary Table 3).

Discussion

We have found that FENO levels were about 21% higher in males than females in this large European multicentre study of healthy, middle-aged subjects. Similar determinants of FENO were found to be associated with higher FENO levels in both males and females with increased height and IgE sensitization. Current smoking was found to be associated with lower FENO levels and the size of this effect was larger in men than women. Higher age related to higher FENO levels, and this effect was seen at a lower age in women than men. Previous smoking was related to a small, but significant decrease of FENO levels in men.

In our study, FENO levels were 21% higher in men than women (18.2 vs15.0 ppb). Several previous studies have also reported an association between increased FENO levels and male sex(20-23). Kim *et al.*(22) reported, based on data from 166 healthy Korean adults (aged 20–68 years), that men had 27% higher FENO levels than women (35.7 vs 26.0 ppb). Taylor *et al.*(23) studied 895 healthy adults at age 32 years and found that men had approximately 25% higher FENO levels than women (15.5 vs 11.6 ppb). A similar size of the sex difference has also been reported in asthmatic subjects(21, 24) – for example, Al-shamkhi *et al.*(21) reported that in 557 subjects with asthma from the Swedish GA2LEN study, men had 32% higher FENO levels than women (24.0 vs 16.4 ppb). However, not all studies have been able to detect sex differences of FENO(25, 26). Olin *et al.*(25) studied 2,200 randomly selected healthy adults, aged 25 to 75 years, and reported that sex was not

independently associated with FENO. In a recent study by Högman *et al.*(26) on 433 healthy subjects, age 7–78 years, a significant sex effect on FENO levels could be reported only for the middle age group, 20–49 years. The mechanism of how sex affects FENO is not fully understood, but a few hypotheses are worth mentioning. Greater height(27) could explain larger lung and airway size, leading to larger surface of the airway mucosa, larger airway calibre, and increased NO release(20, 28). However, this could only partly explain our differences, as male sex was still associated with increased FENO level after adjustment for height. Other potential mechanisms might be genetic differences(29) and effects of oestrogen(30, 31).

Our study showed a significantly increased level of FENO after age 55 in women and age 60 in men. Jacinto *et al.*(11) reported an increase in FENO level in the age group 14–16 years, depending on sex, based on data from the National Health and Nutrition Examination Survey (NHANES). Beyond this age, FENO plateaus and shows stable values until age 45 years in women and age 59 years in men, when it starts to increase. There were some differences between our study and the study of Jacinto *et al.* regarding studied population, as Jacinto *et al.* excluded subjects with hay fever, previous/current smoking, and suspicion of inflammatory diseases. These changes in FENO seem related to somatic growth in childhood, which ends in the upper teens. The increase from middle age and up may be primarily related to structural changes in the lungs, for example loss of alveolar elastic recoil and alveolar surface area(32) and reduced alveolar capillary diffusion of NO(26, 33). This process probably starts earlier in women than men and to some extent explains the present findings and the findings of Jacinto *et al.* However, a recent study on 303 healthy, non-smoking seniors, aged over 65 years (with a mean age of 85 years), found

no difference in F_ENO values between males and females(34), so a cohort study covering all ages would be of interest to fully understand the relation of F_ENO to age and sex.

In the present study, current and ex-smokers showed lower F_ENO levels compared with never-smokers. This result is in accordance with a study of Xu *et al.* on 11,160 subjects from NHANES; they showed that active smoking, measured by self-report, among healthy and asthmatic subjects, was associated with 37% and 45% lower F_ENO levels, respectively(35). In the present study, there was a significant interaction between sex and current smoking. However, this is likely to be due to the fact that men smoked more, as no interaction with sex on F_ENO levels was found in relation to number of smoked cigarettes. This argues against the idea that women might be more sensitive to smoking in terms of F_ENO reduction.

Further, there was a relation between previous smoking and F_ENO in men in form of a 5% reduction in F_ENO levels. Other authors diverge on this matter, reporting a decrease(36), no effect(25, 37), or an increase of F_ENO levels(38). We also found that in men there was an association with time since they stopped smoking, suggesting that the decrease might be seen among all men who stopped smoking recently. However, this effect was not found in women. Several mechanisms on the link of current smoking with decreased F_ENO have been proposed: down-regulation of enzymatic NO formation in the bronchial compartment, as well as in the oropharyngeal compartment(39). Interferon gamma, which is present in normal airways, seem to be down-regulated in smokers, which leads to a decreased expression of iNOS in the human respiratory epithelium(39); another potential mechanism is that smoke contains high levels of NO, which has been found to have an inhibitory feedback effect on NOS(40).

Allergic sensitization was associated with higher levels of FENO, especially when subjects were sensitized to both grass and mite and/or cat allergens. IgE sensitization has in other studies been shown to be related to higher FENO levels(14, 38) and a degree of IgE sensitization has been reported to relate to FENO levels, either when assessed as titres of IgE(14) or number of sensitizations/types of allergens. In a study by Yau *et al.* on 1,321 healthy children, significant positive associations were found between FENO and specific allergens, and between FENO and the number of sensitizations. However, Silvestri *et al.* studied 112 children with stable, mild intermittent asthma and no differences were seen between FENO levels and mono- and poly-sensitized subjects(41).

We found no significant association between BMI and FENO levels in the multivariate model. These results indicate that BMI has no effect on FENO levels, and that FENO is affected to a greater extent by confounders such as sex, age, and height. Our results are in accordance with a study by Kim *et al.,* a cross-sectional study on 117 healthy subjects, aged 20–68 years, which could not find any significant association between BMI and FENO(42). Similar results were also seen in a study on healthy children(43). However, some studies have shown contradictory results. Studies by De Winter-de Groot *et al.*(44), on 24 healthy non-smoking subjects, and by Kazaks *et al.*(45), on 25 healthy subjects, reported a significant positive association between BMI and FENO.

The main strength of the current report is the use of a large, multicentre, general population sample with high quality and standardized measurements of exhaled NO, using the same type of device in all centres. Nevertheless, some limitations must be taken into consideration. ECRHS III does not include measures of bronchial responsiveness. We

excluded subjects with self-reported asthma and/or asthma symptoms in the 12 months preceding the questionnaire. Thus, it is possible that subjects with no or minimal symptoms in the most recent 12 months might be enrolled as healthy subjects. However, subjects receiving medication were excluded, which would argue against subjects with asthma having been enrolled as healthy subjects. Also, having subjects from different centres and geographical areas is a strength indicating that these findings could be valid in the general population. The present population is recruited from the random sample of ECRHS. However due the long-term follow-up time and this is a second follow up, so selection bias can´t be ruled out.

Our data confirm a difference in F_ENO levels between men and women. Present algorithm for clinical interpretation F_ENO do not take sex in to account. F_ENO started increasing at lower age in women than in men, suggesting that interpretation of F_ENO levels in adults aged over 50 years should take into account both age and sex. Similar determinants and effect sizes of different confounders such as current smoking and IgE sensitization could also be found for both men and women. The absolute effect size in this study was not very large, so further studies need to establish if and how to incorporate this information in clinical practice.

Abbreviation

ATS	American Thoracic Society
BMI	Body mass index
ECRHS	European Community Respiratory Health Survey
F_ENO	Fractional exhaled nitric oxide
IgE	Immunoglobulin E

NO Nitric oxide

NOS Nitric oxide synthases

References

1. Guo FH, Uetani K, Haque SJ, Williams BR, Dweik RA, Thunnissen FB, et al. Interferon gamma and interleukin 4 stimulate prolonged expression of inducible nitric oxide synthase in human airway epithelium through synthesis of soluble mediators. The Journal of clinical investigation. 1997;100(4):829-38.
2. Shrestha SK, Drews A, Sharma L, Pant S, Shrestha S, Neopane A. Relationship between total serum immunoglobulin E levels, fractional exhaled breath Nitric Oxide levels and absolute blood eosinophil counts in atopic and non-atopic asthma: a controlled comparative study. Journal of breath research. 2017.
3. Coumou H, Bel EH. Improving the diagnosis of eosinophilic asthma. Expert review of respiratory medicine. 2016;10(10):1093-103.
4. Neelamegan R, Saka V, Tamilarasu K, Rajaram M, Selvarajan S, Chandrasekaran A. Clinical Utility of Fractional exhaled Nitric Oxide (FeNO) as a Biomarker to Predict Severity of Disease and Response to Inhaled Corticosteroid (ICS) in Asthma Patients. Journal of clinical and diagnostic research : JCDR. 2016;10(12):Fc01-fc6.
5. Dressel H, de la Motte D, Reichert J, Ochmann U, Petru R, Angerer P, et al. Exhaled nitric oxide: independent effects of atopy, smoking, respiratory tract infection, gender and height. Respiratory medicine. 2008;102(7):962-9.
6. Kumar R, Gupta N. Exhaled nitric oxide atopy, and spirometry in asthma and rhinitis patients in India. Advances in respiratory medicine. 2017;85(4):186-92.
7. Rouhos A, Ekroos H, Karjalainen J, Sarna S, Haahtela T, Sovijarvi AR. Smoking attenuates increase in exhaled nitric oxide in atopic but not in nonatopic young adults with asthma. International archives of allergy and immunology. 2010;152(3):226-32.
8. Murata A, Kida K, Hasunuma H, Kanegae H, Ishimaru Y, Motegi T, et al. Environmental influence on the measurement of exhaled nitric oxide concentration in school children: special reference to methodology. Journal of Nippon Medical School = Nippon Ika Daigaku zasshi. 2007;74(1):30-6.
9. Evjenth B, Hansen TE, Holt J. Exhaled nitric oxide decreases during exercise in non-asthmatic children. The clinical respiratory journal. 2013;7(2):121-7.
10. Nguyen DT, Kit BK, Brody D, Akinbami LJ. Prevalence of high fractional exhaled nitric oxide among US youth with asthma. Pediatric pulmonology. 2017;52(6):737-45.
11. Jacinto T, Malinovschi A, Janson C, Fonseca J, Alving K. Evolution of exhaled nitric oxide levels throughout development and aging of healthy humans. Journal of breath research. 2015;9(3):036005.
12. Burney PG, Luczynska C, Chinn S, Jarvis D. The European Community Respiratory Health Survey. The European respiratory journal. 1994;7(5):954-60.
13. Janson C, Anto J, Burney P, Chinn S, de Marco R, Heinrich J, et al. The European Community Respiratory Health Survey: what are the main results so far? European Community Respiratory Health Survey II. The European respiratory journal. 2001;18(3):598-611.

14. Malinovschi A, Janson C, Holmkvist T, Norback D, Merilainen P, Hogman M. IgE sensitisation in relation to flow-independent nitric oxide exchange parameters. Respiratory research. 2006;7:92.
15. Cerveri I, Cazzoletti L, Corsico AG, Marcon A, Niniano R, Grosso A, et al. The impact of cigarette smoking on asthma: a population-based international cohort study. International archives of allergy and immunology. 2012;158(2):175-83.
16. ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005. American journal of respiratory and critical care medicine. 2005;171(8):912-30.
17. Kapande KM, McConaghy LA, Douglas I, McKenna S, Hughes JL, McCance DR, et al. Comparative repeatability of two handheld fractional exhaled nitric oxide monitors. Pediatric pulmonology. 2012;47(6):546-50.
18. Jacinto T, Alving K, Correia R, Costa-Pereira A, Fonseca J. Setting reference values for exhaled nitric oxide: a systematic review. The clinical respiratory journal. 2013;7(2):113-20.
19. Fitzmaurice G. M, Laird N. M, Ware JH. Applied longitudinal analysis (2012).
20. Olivieri M, Talamini G, Corradi M, Perbellini L, Mutti A, Tantucci C, et al. Reference values for exhaled nitric oxide (reveno) study. Respiratory research. 2006;7:94.
21. Al-Shamkhi N, Alving K, Dahlen SE, Hedlin G, Middelveld R, Bjerg A, et al. Important non-disease-related determinants of exhaled nitric oxide levels in mild asthma - results from the Swedish GA(2) LEN study. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2016;46(9):1185-93.
22. Kim SH, Kim TH, Sohn JW, Yoon HJ, Shin DH, Park SS. Reference values and determinants of exhaled nitric oxide in healthy Korean adults. The Journal of asthma : official journal of the Association for the Care of Asthma. 2010;47(5):563-7.
23. Taylor DR, Mandhane P, Greene JM, Hancox RJ, Filsell S, McLachlan CR, et al. Factors affecting exhaled nitric oxide measurements: the effect of sex. Respiratory research. 2007;8:82.
24. Gemicioglu B, Musellim B, Dogan I, Guven K. Fractional exhaled nitric oxide (FeNo) in different asthma phenotypes. Allergy & rhinology (Providence, RI). 2014;5(3):157-61.
25. Olin AC, Rosengren A, Thelle DS, Lissner L, Bake B, Toren K. Height, age, and atopy are associated with fraction of exhaled nitric oxide in a large adult general population sample. Chest. 2006;130(5):1319-25.
26. Hogman M, Thornadtsson A, Liv P, Hua-Huy T, Dinh-Xuan AT, Tufvesson E, et al. Effects of growth and aging on the reference values of pulmonary nitric oxide dynamics in healthy subjects. Journal of breath research. 2017;11(4):047103.
27. Jilma B, Kastner J, Mensik C, Vondrovec B, Hildebrandt J, Krejcy K, et al. Sex differences in concentrations of exhaled nitric oxide and plasma nitrate. Life sciences. 1996;58(6):469-76.
28. Malmberg LP, Petays T, Haahtela T, Laatikainen T, Jousilahti P, Vartiainen E, et al. Exhaled nitric oxide in healthy nonatopic school-age children: determinants and height-adjusted reference values. Pediatr Pulmonol. 2006;41(7):635-42.
29. Lund MB, Kongerud J, Nystad W, Boe J, Harris JR. Genetic and environmental effects on exhaled nitric oxide and airway responsiveness in a population-based sample of twins. The European respiratory journal. 2007;29(2):292-8.
30. Scichilone N, Battaglia S, Braido F, Collura A, Menoni S, Arrigo R, et al. Exhaled nitric oxide is associated with cyclic changes in sexual hormones. Pulmonary pharmacology & therapeutics. 2013;26(6):644-8.
31. Mandhane PJ, Hanna SE, Inman MD, Duncan JM, Greene JM, Wang HY, et al. Changes in exhaled nitric oxide related to estrogen and progesterone during the menstrual cycle. Chest. 2009;136(5):1301-7.

32. Levitzky MG. Effects of aging on the respiratory system. The Physiologist. 1984;27(2):102-7.
33. Gelb AF, George SC, Camacho F, Fraser C, Flynn Taylor C, Shakkottai S. Increased nitric oxide concentrations in the small airway of older normal subjects. Chest. 2011;139(2):368-75.
34. Malerba M, Damiani G, Carpagnano GE, Olivini A, Radaeli A, Ragnoli B, et al. Values in Elderly People for Exhaled Nitric Oxide Study. Rejuvenation research. 2016;19(3):233-8.
35. Xu X, Hu H, Kearney GD, Kan H, Carrillo G, Chen X. A population-based study of smoking, serum cotinine and exhaled nitric oxide among asthmatics and a healthy population in the USA. Inhalation toxicology. 2016;28(14):724-30.
36. Malinovschi A, Janson C, Holmkvist T, Norback D, Merilainen P, Hogman M. Effect of smoking on exhaled nitric oxide and flow-independent nitric oxide exchange parameters. The European respiratory journal. 2006;28(2):339-45.
37. Jacinto T, Malinovschi A, Janson C, Fonseca J, Alving K. Differential effect of cigarette smoke exposure on exhaled nitric oxide and blood eosinophils in healthy and asthmatic individuals. Journal of breath research. 2017;11(3):036006.
38. Thorhallsdottir AK, Gislason D, Malinovschi A, Clausen M, Gislason T, Janson C, et al. Exhaled nitric oxide in a middle-aged Icelandic population cohort. Journal of breath research. 2016;10(4):046015.
39. Marteus H, Mavropoulos A, Palm JP, Ulfgren AK, Bergstrom J, Alving K. Nitric oxide formation in the oropharyngeal tract: possible influence of cigarette smoking. Nitric oxide : biology and chemistry. 2004;11(3):247-55.
40. Balint B, Donnelly LE, Hanazawa T, Kharitonov SA, Barnes PJ. Increased nitric oxide metabolites in exhaled breath condensate after exposure to tobacco smoke. Thorax. 2001;56(6):456-61.
41. Silvestri M, Sabatini F, Spallarossa D, Fregonese L, Battistini E, Biraghi MG, et al. Exhaled nitric oxide levels in non-allergic and allergic mono- or polysensitised children with asthma. Thorax. 2001;56(11):857-62.
42. Kim SH, Kim TH, Lee JS, Koo TY, Lee CB, Yoon HJ, et al. Adiposity, adipokines, and exhaled nitric oxide in healthy adults without asthma. The Journal of asthma : official journal of the Association for the Care of Asthma. 2011;48(2):177-82.
43. Kovesi T, Kulka R, Dales R. Exhaled nitric oxide concentration is affected by age, height, and race in healthy 9- to 12-year-old children. Chest. 2008;133(1):169-75.
44. De Winter-de Groot KM, Van der Ent CK, Prins I, Tersmette JM, Uiterwaal CS. Exhaled nitric oxide: the missing link between asthma and obesity? The Journal of allergy and clinical immunology. 2005;115(2):419-20.
45. Kazaks A, Uriu-Adams JY, Stern JS, Albertson TE. No significant relationship between exhaled nitric oxide and body mass index in people with asthma. The Journal of allergy and clinical immunology. 2005;116(4):929-30; author reply 30.

Tables

Table 1. Baseline characteristics of participants divided by whole sample, men and women

Variable		**Men**	**Women**
		(n = 1,969)	**(n = 1,912)**
F_ENO (ppb)* (geometric), 95% CI		18.2 (17.7, 18.6)	15.0 (14.7, 15.4)
Age (years)		54.4 (±7.1)	53.9 (±7.0)
Age (quartile), n (%)			
Q1: 39.7–48.3 years		475 (24.1)	504 (26.4)
Q2: 48.4–54.5 years		477 (24.2)	510 (26.7)
Q3: 54.6–60.2 years		505 (25.7)	448 (23.4)
Q4: 60.3–67.6 years		512 (26.0)	450 (23.5)
Height (m)		1.77 (±0.07)	1.64 (±0.07)
Weight (kg)		85.7 (±14.6)	70.9 (±14.4)
BMI (kg/m_2), n (%)			
< 18.5		7 (0.4)	21 (1.1)
>18.5 to 25		585 (29.8)	857 (44.9)
>25 to 30		956 (48.6)	630 (33.0)
>30 to 35		332 (16.9)	272 (14.3)
≥ 35		86 (4.4)	127 (6.7)
Total IgE (kU/L) (geometric), 95% CI		28.3 (26.4, 30.4)	20.6 (19.1, 22.2)
IgE sensitized to different allergen, n (%):			
Non-sensitized		1.467 (77.5)	1.482 (81.0)
Sensitized only to grass pollen		132 (7.0)	130 (7.1)
Sensitized only to perennial allergens		188 (9.9)	127 (7.0)
Sensitized both to grass pollen and perennial allergens		107 (5.6)	90 (4.9)
Hay fever, yes, n (%)		478 (24.3)	536 (28.1)
Smoking:	Never-smoker	782 (39.8)	898 (47.1)
n (%)	Ex-smoker	798 (40.6)	681 (35.8)
	Current smoker	386 (19.6)	326 (17.1)

Current smokers:	Number cig/day	11.0 (10.0, 12.1)	8.2 (7.4, 9.1)
(geometric mean, 95% CI)	Pack-years	25.7 (23.6, 27.9)	18.0 (16.4, 19.4)
Ex-smokers (±SD)	Time since stopped	17.7 (±11.6)	18.4 (±11.1)
(geometric mean, 95% CI)	smoking (year)		
	Pack-years	8.8 (8.0, 9.8)	6.9 (6.3, 7.6)

Values: n [%] and mean [±SD]. Perennial allergens (mite- and/or cat-positive). Abbreviations: BMI: body mass index, F_ENO: fractional exhaled nitric oxide, IgE: immunoglobulin E.

Table 2. Bivariate linear regression analysis, (β-coefficient**,** 95% CI) between FENO and ex-smokers time since stopped smoking/ex-packs per year and in smokers no. cigarettes per day/current packs per year, both by sex.

logFENO		**Men**		**Women**	
		β-coefficient (95% CI)	**p value**	**β-coefficient (95% CI)**	**p value**
Ex-smokers:	**Time since stopped smoking/10 years**	1.05 (1.02, 1.08)	0.004	1.02 (0.99, 1.05)	0.33
	log Ex packs/year	0.01 (-0.02, 0.04)	0.44	-0.004 (-0.04, 0.03)	0.80
Smokers:	**log No. cigarettes/day**	-0.16 (-0.23, -0.11)	< 0.001	-0.12 (-0.18, -0.06)	< 0.001
	log Current packs/year	-0.21 (-0.28, -0.15)	< 0.001	-0.11 (-0.17, -0.04)	0.001

FENO and time since stopped smoking in ex-smokers has been back-transformed. Example: β-coeff. = 1.05 means that subjects who have quit smoking have 5% higher FENO levels per 10 years. When both dependent and independent variable were log transform, the β-coefficient should be interpreted as the % change of FENO when the independent variable changes 1 % (e.g., 1% increase in cigarettes/day leads to 0.16 % decreased FENO levels). Abbreviations: FENO: fractional exhaled nitric oxide, CI: confidence interval.