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PAPER

Evolution of exhaled nitric oxide levels throughout development and aging of healthy humans

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21 May 2015Tiago Jacinto^{1,2,6}, Andrei Malinovski³, Christer Janson⁴, João Fonseca^{1,2} and Kjell Alving⁵¹ Instituto & Hospital CUF Porto, Portugal² CINTESIS, Faculdade de Medicina da Universidade do Porto, Portugal³ Department of Medical Sciences: Clinical Physiology, Uppsala University, Sweden⁴ Department of Medical Sciences: Respiratory Medicine and Allergology, Uppsala University, Sweden⁵ Department of Women's and Children's Health, Uppsala University, SwedenE-mail: tiagojacinto@med.up.pt**Keywords:** exhaled nitric oxide, aging, healthy, physiologySupplementary material for this article is available [online](#)**Abstract**

It is not fully understood how the fraction of exhaled nitric oxide (FeNO) varies with age and gender in healthy individuals. We aim to describe the evolution of FeNO with age, giving special regard to the effect of gender, and to relate this evolution to natural changes in the respiratory tract.

We studied 3081 subjects from NHANES 2007–08 and 2009–10, aged 6–80 years, with no self-reported diagnosis of asthma, chronic bronchitis or emphysema, and with normal values of blood eosinophils and C-reactive protein. The relationship of the mean values of FeNO to age, in all participants and divided by gender, was computed, and compared with changes in anatomic dead space volume and forced vital capacity. A change-point analysis technique and subsequent piecewise regression was used to detect breakpoints in the evolution of FeNO with age.

Three distinct phases in the evolution of FeNO throughout the age range 6–80 years can be seen. FeNO values increase linearly between 6–14 years of age in girls and between 6–16 years of age in boys, in parallel with somatic growth. After that, FeNO levels plateau in both genders until age 45 years in females and age 59 years in males, when they start to increase linearly again. This increase continues until age 80.

Our data clearly show a triphasic evolution of FeNO throughout the human age range in healthy individuals. This should be accounted for in development of reference equations for normal FeNO values.

Introduction

The measurement of the fraction of exhaled nitric oxide (FeNO) is an accurate, reproducible and non-invasive diagnostic test for inflammatory airway disease [1]. Airway NO originates primarily in respiratory epithelial cells and is produced by the inducible nitric oxide synthase (iNOS) [2]. Besides environmental factors such as allergens and cigarette smoke exposure, exhaled NO is known to be affected by some individual characteristics, such as age, height and gender [3].

However, it is not yet fully understood how FeNO varies with age and gender in healthy individuals. The analysis of datasets from large population-based observational studies, such as the National Health and Nutrition Examination Survey (NHANES) can provide a much needed insight into these variations.

Exhaled NO is determined by the airway NO production and subsequent release into the airway lumen as well as the steady-state levels of NO in the alveoli, according to the two-compartment model [4, 5]. Exhaled NO increases with age in childhood [6, 7] and this increase is assumed to reflect the growth of the lungs with concurrent increase in the airway mucosal surface area from which NO can diffuse [8]. However, studies using standardized methods performed in children so far have been relatively small and gender differences have not yet been identified. Levels of FeNO have also been shown to increase with

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age in adulthood, and the relationship has been anticipated to be linear [9]. The reason for this increase is unclear, but NO diffusion changes at the alveolar level have been implicated [10].

The mucosal formation of NO requires a series of biochemical reactions that are dependent on energy, a complex enzyme, and several cofactors and substrates [2, 14]. The production of NO by iNOS in the airways is a stable and strictly regulated process in homeostasis, already beginning in utero [11, 12], and remains constant even after high doses of systemic corticosteroids in healthy individuals [13, 14]. Disregarding confounder factors such as cigarette smoking, rhinovirus infections and nitrate intake [3], it may thus be hypothesized that FeNO in healthy subjects should primarily be affected by functional changes in the different NO exchange compartments, which, in turn, are mostly affected by age. We suggest that the changes observed in lung function throughout normal human development and aging [15] are closely linked to FeNO.

In this study, we aim to describe the evolution of FeNO with age in 2007–10 NHANES subjects 6–80 years old. The analysis is hypothesis-driven as indicated above, and will thus be divided by gender and related to natural changes in the respiratory tract.

Methods

The National Health and Nutrition Examination Survey (NHANES) is a nationally representative series of studies designed to assess the health and nutritional status of adults and children in the United States, combining interviews and physical examinations and conducted by the National Center for Health Statistics (NCHS), a part of the Centers for Disease Control and Prevention (CDC). More detailed info is available at: www.cdc.gov/nchs/nhanes/about_nhanes.htm.

From a total of 20 686 participants in NHANES 2007–10, we included healthy individuals defined as having no previous diagnosis of asthma, emphysema or chronic bronchitis, no hay fever in the past 12 months and having smoked less than 100 cigarettes earlier in life. Furthermore, to exclude possible inflammatory diseases that may affect FeNO values, we excluded individuals with blood eosinophil (B-Eos) count over 300 cells mm⁻³ and serum C-reactive protein over 1 mg dL⁻¹ [16].

Exhaled NO

FeNO was measured with an electrochemical analyzer (NIOX MINO; Aerocrine AB, Solna, Sweden), before performing spirometry. Quality control checks were performed weekly. Measurements were performed in specially-designed and equipped mobile centers. All participants age 6–79 years were eligible for FeNO measurement. Patients that had a breathing problem requiring the use of supplemental oxygen during the day and/or with pain or a physical problem that prevented deep breaths and forceful exhalations were excluded from measuring FeNO. Participants measured

FeNO in a seated position, in front of a mirror, to facilitate the maneuver, which consisted in emptying the lungs, putting the mouth over the filter, while sealing the lips tightly around it, taking a deep breath until the lungs are filled and then breathing out at a normal and constant rate through the filter. A detailed description of the methods is available at: www.cdc.gov/nchs/data/nhanes/nhanes_11_12/Respiratory_Health_ENO_Procedures_Manual.pdf.

A valid measurement was defined in accordance with ATS/ERS guidelines [17] as: having two reproducible measurements at an expiratory flow rate of 50 ml s⁻¹, no use of oral or inhaled steroids in the past 2 d and no cough, cold or respiratory illnesses in the past 7 d, no breathing problems requiring oxygen, no problem taking deep breaths, no strenuous exercise in the hour prior to the measurement and no consumption of NO-rich vegetables and meats. Up to four FeNO measurement attempts were made and the mean of two reproducible FeNO measurements (within 2 ppb if levels were <30 ppb or within 10% if levels were >30 ppb) was taken as the final result. The lower and upper detection limit of NIOX MNO is 5 and 300 ppb, respectively. If two measurements were below the limit of detection of the device, a value of 3.5 ppb (lower limit of detection divided by the square root of two) was used as the mean.

Age

The age of each participant at the time of examination was recorded in months. For this study, age was rounded to years, with one decimal place. Moreover, due to the oversampling of adolescents in the NHANES dataset, age was categorized as a single year up to age 21 years, and then in two-year categories until age 80 years, to increase statistical power in the latter ages. This was done to have at least 40 individuals per age category.

Two age groups were defined as follows: developing individuals (age 6–21 years) and developed individuals (age >22 years), following the natural growth and development of the lungs [15].

Lung function

Spirometry was performed following ATS/ERS recommendations [18], with an Ohio 822/827 dry-rolling seal volume spirometer, calibrated before the start of each session. The exclusion criteria for spirometry were one of the following: current painful ear infection, eye surgery in the last 3 months, chest/abdominal surgery in the last 3 months, tuberculosis exposure, history of aneurysm or collapsed lung, history of detached retina, stroke or heart attack in the last 3 months and history of coughing up blood in the last month. Participants were asked to forcefully blow the air out of their lungs for a minimum of 6 s. A minimum of 3 acceptable and reproducible measurements were obtained. FEV₁ and FVC were recorded in milliliters. A detailed description of the methods is available at: www.cdc.gov/nchs/data/nhanes/nhanes_11_12/Spirometry_Procedures_Manual.pdf. Predicted values

for lung function accounting for age, gender, height and race were calculated [19].

Ethics

All protocols were approved by the Ethics Review Board of the National Center for Health Statistics Research. All participants provided written informed consent.

Analysis

We have analyzed the relationship of the mean values of FeNO with age, in all participants and divided by gender, using error bar charts with the mean and 95% confidence intervals of FeNO per age category. This analysis was performed with natural log-transformed FeNO, and the results were back-transformed to obtain geometric means. These relationships were also compared with the change in FVC, FEV₁ and the volume of anatomic dead space (VADS). VADS was calculated using the Kerr equation for height [20]. For detecting the number of significant changes of mean FeNO values in the age categories, we used the change-point analysis technique, which has been described in detail elsewhere [21]. Briefly, this technique iteratively uses a combination of cumulative sum charts and bootstrapping to detect changes. For each change, it provides detailed information including a confidence level indicating the likelihood that a change occurred and a confidence interval indicating when the change occurred. This was followed by the calculation of the exact location of a significant change point and the regression modeling in the evolution of FeNO, computed with the statistical technique breakpoint analysis, which tries to fit piecewise terms in regression models where one or more breakpoints are true parameters of the model. Using this method of iterative fitting of linear models bypasses the non-linear and non-differentiable problems [22].

Results

After excluding individuals with age equal to or less than 72 months as well as subjects with no reproducible FeNO measurements ($n = 13,269$), we included 3081 (15% of total) non-smoking individuals, with no history of self-reported respiratory diseases or hay fever in the past 12 months, and with B-Eos count <300 cells mm^{-3} and C-reactive protein <1 mg dL^{-1} . Details of exclusion criteria are shown in figure 1.

Of the included individuals, 1217 (40%) were developing individuals (age <22 years) and 1864 (60%) were adults (age ≥ 22 years). A histogram of age distribution per gender is depicted in figure 1 in the supplementary material (available at stacks.iop.org/JBR/9/036005/mmedia). The participants' characteristics are shown in table 1.

After plotting FeNO against age, and using change-point analysis, we could confirm the existence of two significant change points in the evolution of FeNO, the first being in adolescence and the second in middle-aged individuals. Three distinct phases in the evolution of

FeNO could then be identified (figure 2). All individual FeNO values with loess smoothing curves are depicted in figure 2 in the supplementary material (available at stacks.iop.org/JBR/9/036005/mmedia).

FeNO increased until the age of 14–16 years depending on gender. After this point, FeNO values seemed to stabilize, showing a plateau in the first decades of adulthood. After this plateau phase, an increase was seen starting in middle age. This process continued throughout the age range studied.

When modeling the detected pattern in the evolution of FeNO using breakpoint analysis, it was possible to define segments and precise breakpoints (table 2).

The first breakpoint (BP1) appeared at slightly younger age in females than males (13.9 years versus 15.5 years, respectively). The second breakpoint (BP2) also appeared at younger age in females than males (44.45 years versus 61.26 years, respectively). The mean slopes between BP1 and BP2 were smaller for both genders, when compared to the slopes before BP1 and after BP2. The resulting model of these relationships has been depicted in figure 3.

Relationship to lung development

In developing individuals, FeNO values increase until age 14.1 years in females and age 15.7 years in males. A very similar development is also seen for the VADS (figure 4—panel (A)).

In this age range, VADS has one breakpoint which appears at a younger age in females than in males (13.9 versus 15.5 years) (table 3). The model of these relationships is depicted in panel (B) of figure 4.

In figure 5, it can be observed that the increase in FeNO from middle age onwards is basically mirrored by a decrease in FVC, in both females and males, which continues until the older ages as FeNO continues to increase.

A breakpoint in FVC was also detected, and appears at younger age in females than in males (34.8 versus 48.8 years) (table 3). FEV₁ showed a more continuous decline in adulthood, with no significant break-points (not shown).

Relationship with inflammation

The evolution of B-Eos count with age followed a different pattern than that of FeNO (figure 3 in the supplementary material (available at stacks.iop.org/JBR/9/036005/mmedia)): first decreasing in both males and females up to age 36 and 35 years, respectively, and then increasing throughout the age range studied (table 1 in the supplementary material (available at stacks.iop.org/JBR/9/036005/mmedia)). This breakpoint in the evolution of B-Eos count occurred during the plateau phase of the FeNO evolution with age, with no apparent interrelationship.

Discussion

In this study, we describe the evolution of FeNO with age in females and males, respectively, in a

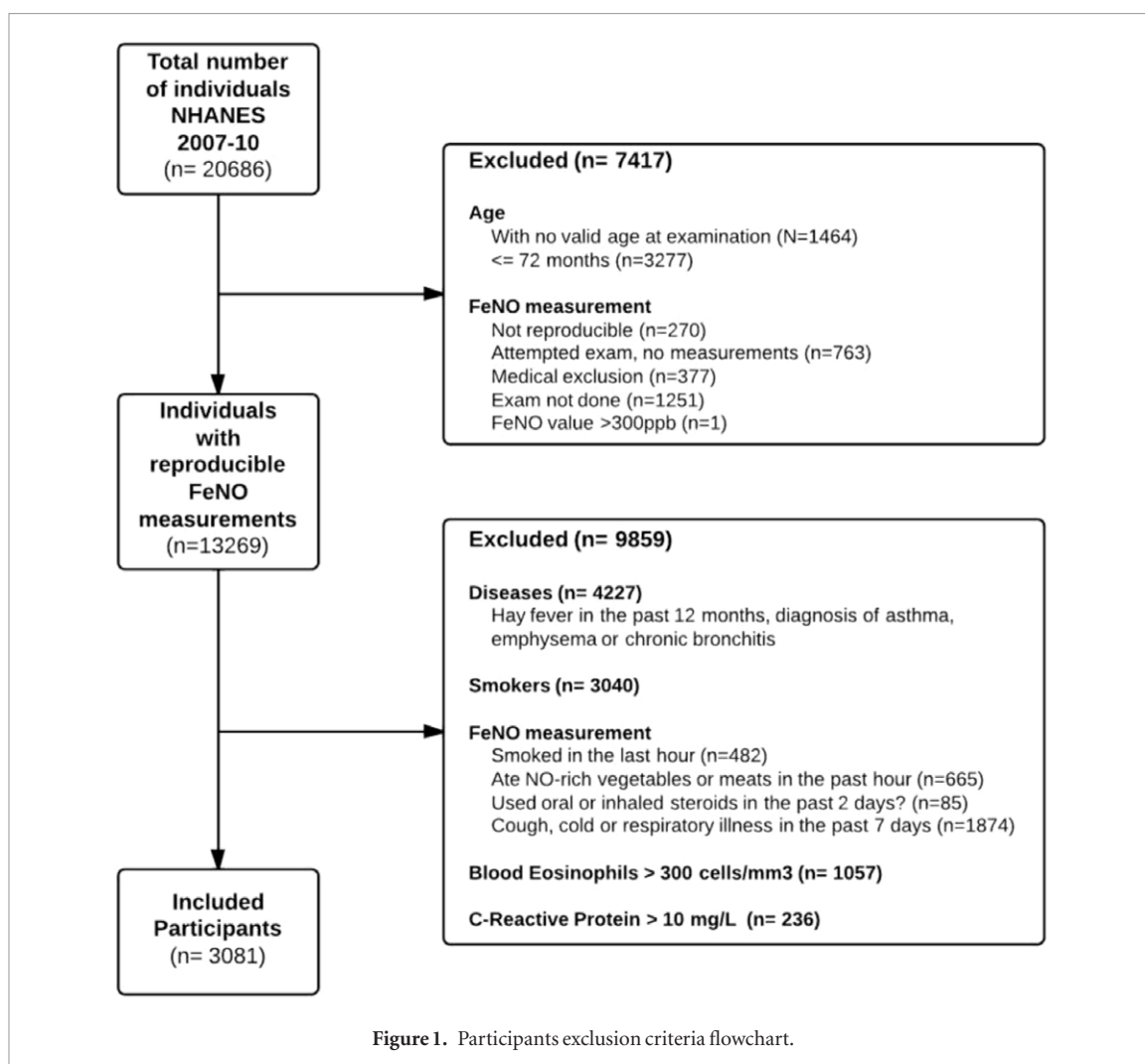


Figure 1. Participants exclusion criteria flowchart.

Table 1. Description of the characteristics of included participants.

	All n = 3081	Developing individuals n = 1217 (40%)	Developed individuals n = 1864 (60%)
Gender			
Male	1,417 (46, 0%)	605 (49.7%)	812 (43.6%)
Female	1,664 (54, 0%)	612 (50.3%)	1,052 (54.4%)
Age (years) ^a	33.8 (20.0)	14.5 (4.1)	46.4 (15.8)
Height (cm) ^a	162.8 (14.3)	157.0 (17.3)	166.5 (10.5)
Weight (kg) ^a	70.7 (23.4)	57.1 (22.8)	79.4 (19.3)
FVC (% predicted) ^a	91.2 (14.3)	94.0 (13.9)	89.4 (14.3)
FEV ₁ (% predicted) ^a	92.8 (14.5)	95.2 (14.4)	91.2 (14.4)
VADS (ml) ^a	89.5 (14.6)	83.6 (17.6)	93.3 (10.6)
B-Eos (cells mm ⁻³) ^b	127 (126–130)	128 (126–130)	129 (126–132)
C-reactive protein (mg dL ⁻¹) ^b	0.9 (0.1–5.9)	0.3 (0.1–4.2)	1.4 (0.2–6.7)
FeNO (ppb) ^b	12.5 (8.5–18.0)	10.0 (7.0–14.5)	14.0 (9.5–20.0)

^a Mean (SD).^b Median (IQR).

large sample of healthy individuals from NHANES 2007–10. We show an increase in FeNO until the age of 14–16 years depending on gender. After this point, FeNO plateaus and shows stable values in the first decades of adulthood. Later on, an increase is seen after age 45 years in women and age 59 years in men, and this process apparently continues into old

age. These changes in FeNO are not mirrored by any age-related changes in systemic inflammation as measured by blood eosinophils. Instead, they seem related to somatic growth in childhood and an accelerated decline in lung function, from middle age and up. Thus, we suggest that the age-related changes of FeNO in healthy individuals are primarily linked

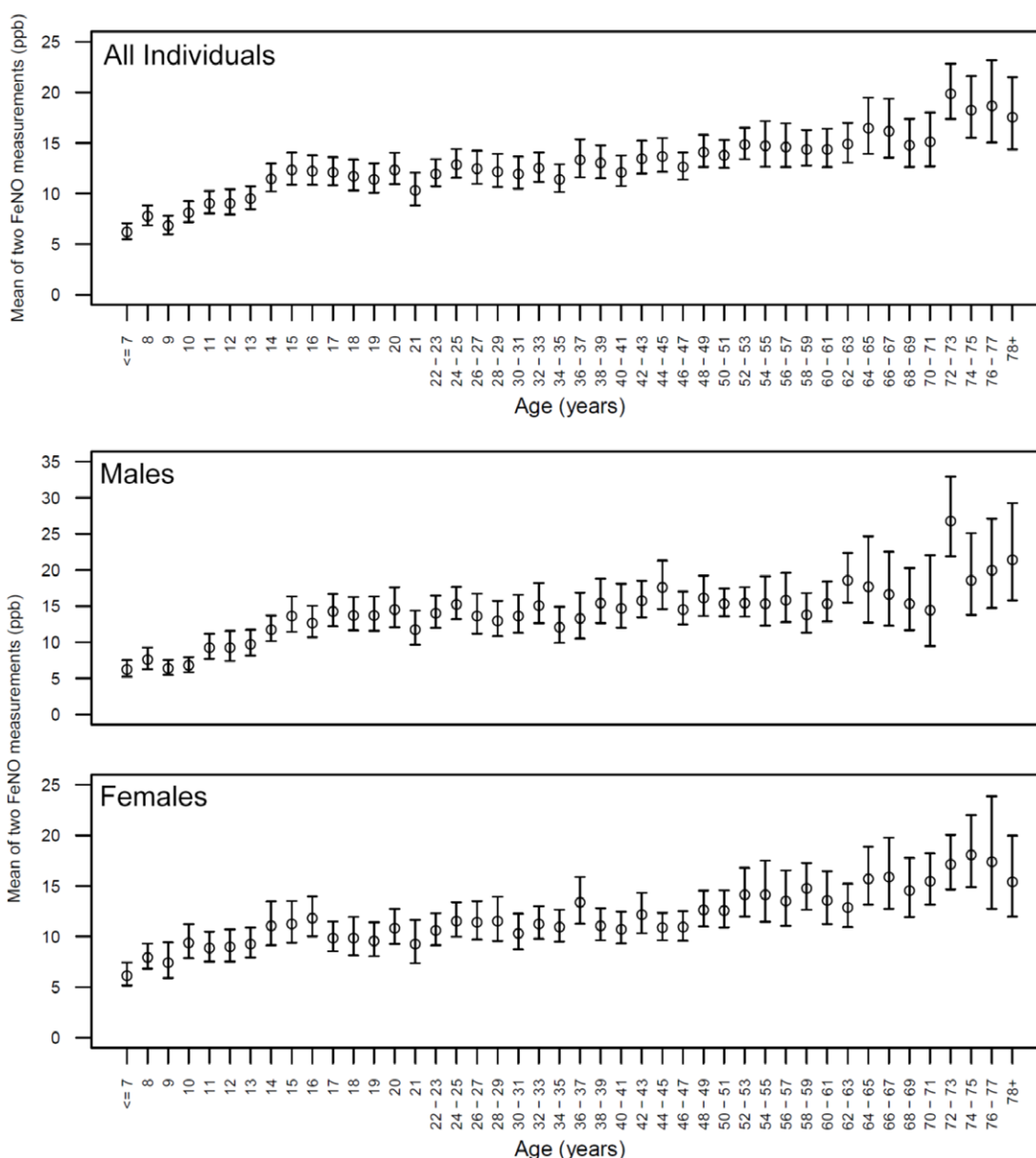


Figure 2. Evolution of FeNO values in the entire age range, in all individuals and divided by gender. FeNO is represented by geometric means and 95% confidence limits for the mean by age category. Geometric means were obtained by the back-transformation of natural logarithm of FeNO.

Table 2. Breakpoint analysis for FeNO values.

	Age breakpoints (years) (95% CI)	Slopes (factor increase per year) (95% CI)		
		Before BP1	Between BP1 and BP2	After BP2
Males				
BP1	15.5 (14.1; 16.9)	1.098 (1.074; 1.123)	1.004 (1.001; 1.007)	1.017 (0.999; 1.034)
BP2	61.3 (47.0; 75.5)			
Females				
BP1	13.9 (11.6; 16.2)	1.064 (1.032; 1.096)	1.004 (1.000; 1.008)	1.012 (1.007; 1.017)
BP2	44.5 (29.9; 59.0)			

BP1: breakpoint 1; BP2: breakpoint 2.

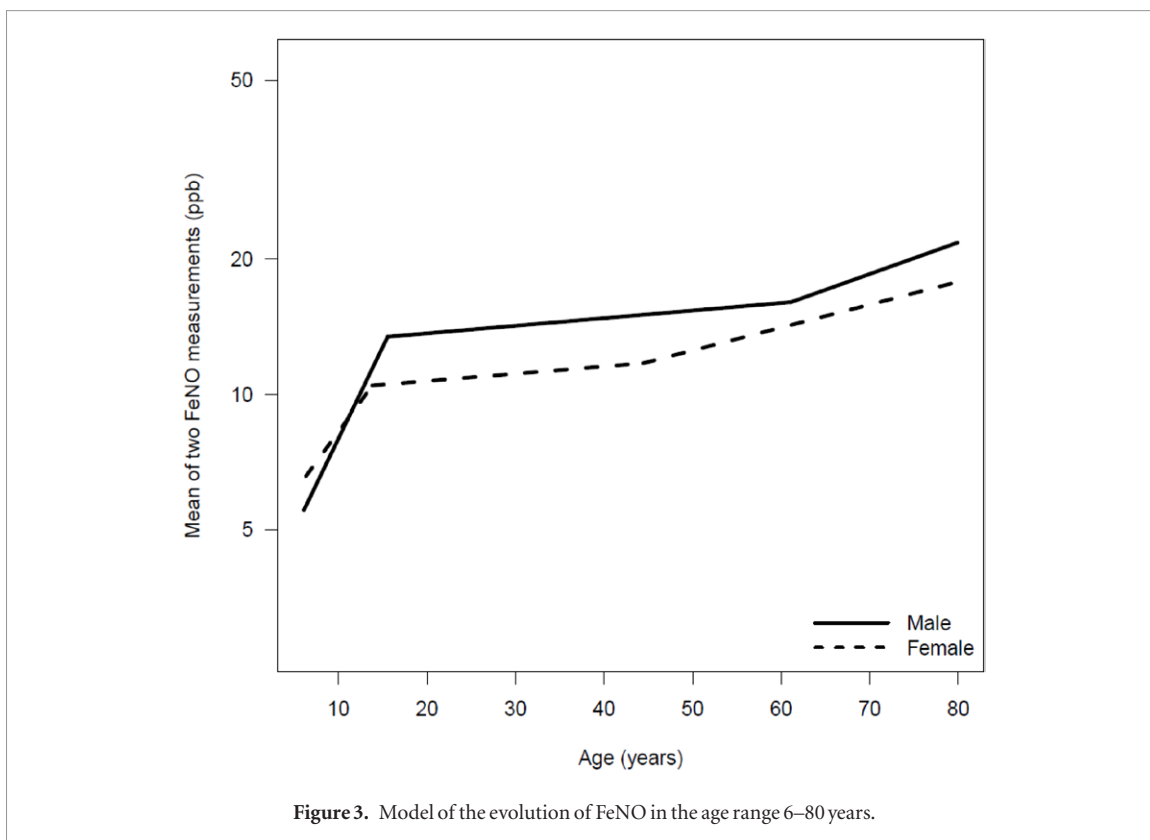


Figure 3. Model of the evolution of FeNO in the age range 6–80 years.

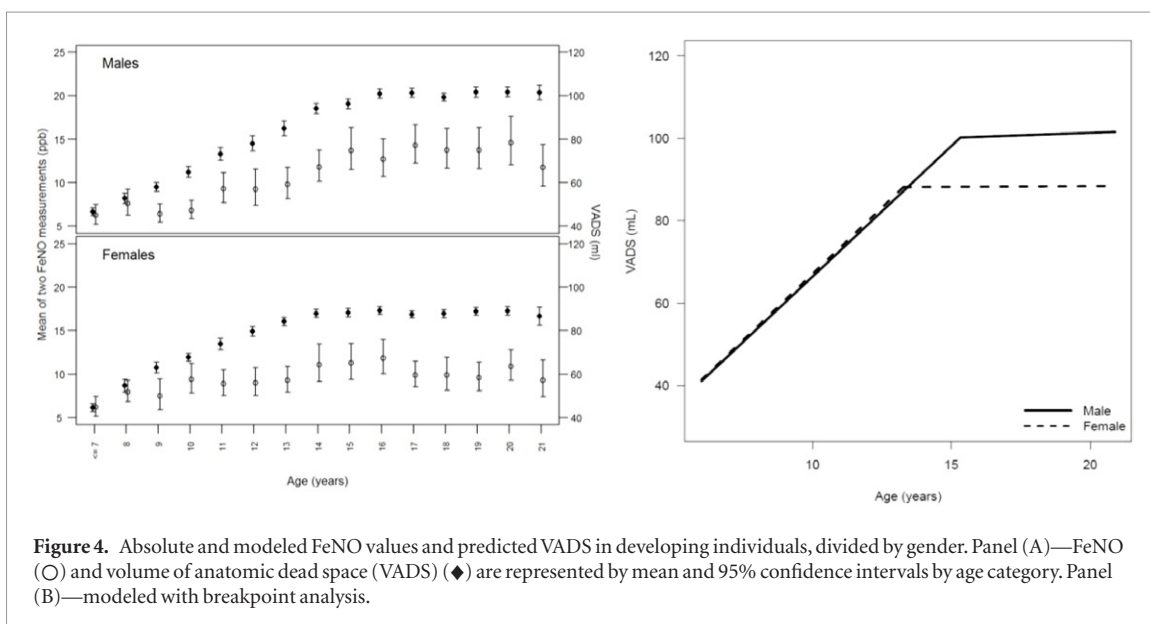


Figure 4. Absolute and modeled FeNO values and predicted VADS in developing individuals, divided by gender. Panel (A)—FeNO (○) and volume of anatomic dead space (VADS) (◆) are represented by mean and 95% confidence intervals by age category. Panel (B)—modeled with breakpoint analysis.

Table 3. Breakpoint analysis for VADS and FVC.

	Age breakpoints (years) (95% CI)	Slopes (95% CI)	
		Before BP	After BP
VADS (Developing individuals)			
Males	15.33 (14.93; 15.74)	6.339 (6.022; 6.657)	0.243 (−0.392; 0.879)
Females	13.28 (12.95; 13.6)	6.425 (6.017; 6.833)	0.034 (−0.296; 0.363)
FVC (Developed individuals)			
Males	48.8 (31.01; 66.58)	−0.019 (−0.029; −0.010)	−0.032 (−0.042; −0.021)
Females	34.81 (30.61; 39.01)	0.002 (−0.013; 0.016)	−0.031 (−0.035; −0.028)

VADS: volume of anatomic dead space; FVC: forced vital capacity; BP: breakpoint.

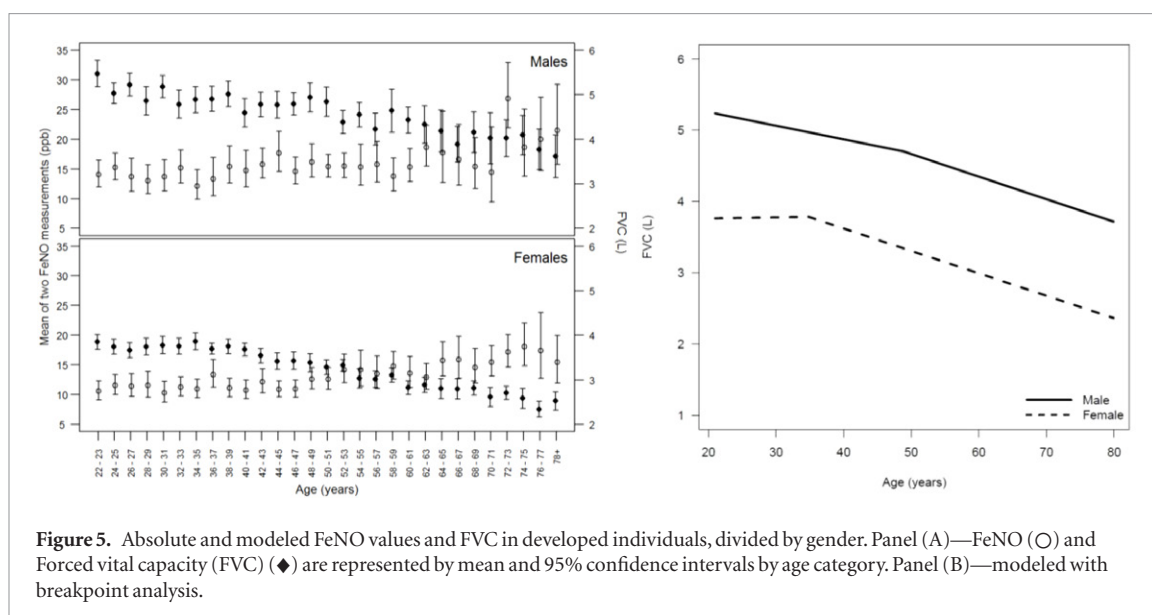


Figure 5. Absolute and modeled FeNO values and FVC in developed individuals, divided by gender. Panel (A)—FeNO (○) and Forced vital capacity (FVC) (◆) are represented by mean and 95% confidence intervals by age category. Panel (B)—modeled with breakpoint analysis.

to the evolution of the structure and function of the respiratory system.

The breakpoint in the increase of FeNO in adolescence appeared to be earlier in females than in males. This mirrors somatic growth, both as generally described and as shown in this material, and consequently the development of the bronchial tree. Supporting this relationship, prematurely born children who develop bronchopulmonary dysplasia show airflow limitation and low levels of exhaled NO at school-age, when compared with healthy children born prematurely [23]. It has previously been shown that estimated anatomic dead space volume correlates with both FeNO and the airway diffusing capacity for NO (DawNO) in healthy schoolchildren [8]. DawNO is probably highly dependent on the total airway mucosal surface area available for NO diffusion, which obviously increases with somatic growth.

A clear-cut biphasic age-dependent evolution in adulthood, with FeNO starting to increase in middle age, was demonstrated in the present study. Several studies have shown that healthy elderly people have elevated FeNO compared with younger adults [10, 24]. The age evolution of FeNO has been poorly studied, but has been anticipated to be linear throughout adulthood [9, 25]. We show a clear plateau phase for FeNO starting at the age of having reached full body height and lasting until middle age. The second increase in FeNO starting in middle age may be related to changes in the alveolar-capillary diffusion of NO as recently discussed by Gelb *et al* [10]. It is well-known that aging is related to structural alterations in the lungs, for example loss of alveolar elastic recoil and alveolar surface area [26]. Loss of alveolar elastic recoil results in increased residual volume and, as a consequence, reduced vital capacity. The reduction in forced vital capacity with age has been shown previously to be biphasic [27] and this is also indicated in our data based on NHANES subjects, with the accelerated decline starting a few years earlier in women than men. Also, the loss of static lung elastic recoil is primarily seen after age 59 years (men and women presented together),

with almost no loss before that age [28]. However, FeNO is probably more directly affected by the pulmonary diffusing capacity for NO, since the rate of NO transfer in the alveoli with binding to hemoglobin will influence the axial diffusion of NO during exhalation [29, 30]. Supporting this view, the diffusing capacity for carbon monoxide (DLCO), as well as alveolar volumes, has been shown to correlate negatively with estimated alveolar NO concentrations, but not bronchial flux, in patients with allergic alveolitis [31]. Both DLCO and alveolar NO were normalized after allergen avoidance in these patients. Unfortunately, FeNO was not presented in this study. However, in a study on healthy volunteers 18–86 years of age, Gelb *et al* showed that alveolar NO and FeNO increase at older age (>59 years) whereas bronchial flux of NO did not change significantly with increasing age [10]. All in all, the results support the view that reduced pulmonary diffusing capacity for NO results in increased alveolar NO and FeNO due to reduced axial diffusion of NO. Thus, the data suggest that FeNO increases with age in adulthood, particularly at ages over 60 years, due to such a decrease in alveolar NO gas transfer rate. This can contribute to increased FeNO both by providing a higher NO concentration in the alveoli at the start of exhalation and by allowing more NO to escape into exhaled breath during exhalation due to reduced axial diffusion [29, 30].

The breakpoint for the increase in FeNO in middle age occurs some 15 years earlier in women than in men. The reduction in FVC throughout adulthood also appeared biphasic in this NHANES material, again with the breakpoint occurring earlier in females, though the breakpoint was not as clear in males as in females. Sherrill *et al* could also confirm a biphasic decline of FVC in women, with the breakpoint at 46 years, but could not detect any breakpoint in men using piecewise regression in a longitudinal study on 930 non-smoking healthy subjects [27]. In the same study, clear-cut breakpoints were identified in upper middle age for both women and men regarding the age-related decrease in

mid-expiratory flows, whereas the reduction in FEV₁ was linear from approximately 25 years of age in this material. Similarly, we could not detect any breakpoint in the FEV₁ decline among the NHANES subjects. Since expiratory flows are thought to better reflect small airways function than FEV₁, these data suggest that the function of small airways show accelerated deterioration starting in middle age. This is probably due to obstruction of acinary airways through the loss of alveolar attachments that stabilize these airways. Furthermore, there is a reduction in the surface of the lung available for gas exchange because of the coalescence of alveoli along with loss of alveolar walls [32]. In line with this, there is a decrease of gas transfer rates and pulmonary capillary volumes with age. This decline has been shown to be biphasic [33, 34]. Interestingly, Aguilaniu *et al* determined the breakpoint for the reduction in lung transfer of CO and NO to age 59 years for men, identical to the breakpoint identified for the increase in FeNO in our study.

Limitations and strengths

This study has some limitations. First, this is an analysis of data from a cross-sectional study that was not specifically designed to address our research question. A second important limitation is the lack of objective assessment of atopy, a factor known to influence FeNO [35, 36]. However, we believe that by removing subjects with intermediate to high B-Eos counts ($>0.3 \times 10^9 \text{L}^{-1}$), the influence of Th2 cytokine-driven mechanisms should be minimal [2]. Furthermore, no marked change in B-Eos count with age could be detected.

The study also has several strengths. This is a large sample of healthy individuals, defined using strict and mainly objective criteria. Moreover, the NHANES study is well-known for the quality of the design; the data available is also checked for inconsistencies and data entry errors. The current study includes a broad age range, including the transition from adolescence to adulthood; previous studies only included children [6] or adults [9], which prohibited detection of the triphasic evolution of FeNO with age.

Conclusion

Our data strongly suggests that there is considerable variation in FeNO values throughout the human age range in healthy individuals, and that this variation is triphasic with gender-specific breakpoints. The evolution of FeNO with age seems related to changes in lung function in connection with growth and aging. During a period of life, between the periods of somatic growth and age-related deterioration in lung function, FeNO is quite stable and only dependent on gender and height in healthy individuals. This should be accounted for in the development of reference equations for FeNO. The official American Thoracic Society clinical practice guidelines on the interpretation of FeNO suggest that FeNO below 25 ppb should be considered normal in

subjects 12 years of age and older, with little likelihood of eosinophilic inflammation [37]. Our data suggest that this cutoff may be too low in older males and too high in younger subjects, especially females, and should be adjusted accordingly. We suggest that segmenting the age range and analyzing genders separately may increase the percentage of explanation from models predicting FeNO in healthy individuals.

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Summary conflict of interest statements

TJ, AM, CJ and JAF have nothing to disclose. KA is an employee and minority shareholder of Aerocrine AB (Sweden).

Author contributions

TJ and KA were responsible for literature search, study design, data analysis, manuscript preparation and review of the manuscript. AM and JF were responsible for study design, data analysis and review of the manuscript. CJ was responsible for review of the manuscript.

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