Eur Respir J 2007; 30: 1074–1081
DOI: 10.1183/09031936.00087807

Smoking is associated with an age-related decline in exhaled nitric oxide

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ABSTRACT: Age-related declines in forced expiratory volume in one second are accelerated in smokers. Smoking is associated with decreased exhaled nitric oxide fraction (*F*eNO). The aim of the present study was to determine the impact of age on *F*eNO in otherwise healthy smokers and nonsmokers.

***F*eNO and serum cotinine levels were measured in 994 healthy subjects aged 18–40 yrs. American Thoracic Society questionnaire data on smoking habits was used to validate serum cotinine levels as a surrogate marker for categorisation of smokers and nonsmokers in the cohort.**

Serum cotinine levels were a good discriminator of smokers (n=99) and nonsmokers (n=895). *F*eNO levels were significantly lower in otherwise healthy smokers compared with nonsmokers. There was an inverse correlation of serum cotinine levels with *F*eNO. No correlation of age with *F*eNO was found in nonsmokers but an inverse correlation of *F*eNO with age in smokers was found. *F*eNO was significantly lower in smokers aged 21–40 yrs compared with nonsmokers aged 21–40 yrs, but was not lower in smokers aged 18–20 yrs compared with nonsmokers of the same age.

Smoking was associated with decreased exhaled nitric oxide. The greatest smoking-related declines in exhaled nitric oxide occurred in older subjects. This suggests that smoking is associated with age-related declines in exhaled nitric oxide and justifies future mechanistic studies that address the impact of exhaled nitric oxide decline on lung function.

KEYWORDS: Ageing, humans, nitric oxide, smoking

Inhaled tobacco smoke has acute and chronic effects on exhaled nitric oxide fraction (*F*eNO). *F*eNO levels are lower in otherwise healthy subjects who habitually smoke tobacco compared with nonsmokers [1–8], and *F*eNO levels decrease in nonsmokers and smokers acutely after smoking a cigarette and after passive smoke exposure [1, 5, 9]. There is an inverse relationship between the number of cigarettes smoked per day and *F*eNO levels [1]. *F*eNO levels increase after smoking cessation but not to normal levels [2, 6, 10], which suggests that smoking-related declines in *F*eNO may be associated with permanent lung damage. As respiratory epithelium is the likely source of most *F*eNO [11–15] and is damaged by chronic smoke exposure [16–20], smoking-related declines in *F*eNO may be a marker of airway epithelial damage.

There are several reasons to believe that low *F*eNO levels in smokers may be detrimental. Ageing is associated with declines in lung function and age-related declines in lung function are accelerated in smokers [21, 22]. Smoking-related declines in forced expiratory volume in one second (FEV_1) with increasing age are important determinants of obstructive lung disease [23, 24]. Nitric oxide (NO) promotes bronchodilation [25–28] and, as such, smoking-related declines in *F*eNO may contribute to bronchospasm or obstructive lung disease in smokers. NO is also important in resistance to infection [29, 30] and decreased *F*eNO may predispose to respiratory infection.

If smoking-related declines in *F*eNO were associated with respiratory epithelial damage, then otherwise healthy older smokers should have lower *F*eNO levels than younger smokers. Prior studies of *F*eNO in nonsmoking adults indicated that there was no association of age with *F*eNO levels [31]. In a recent study, MCSHARRY *et al.* [32] identified an association of decreasing *F*eNO levels with age in asthmatic smokers. Whether smoking results in age-related declines in *F*eNO in otherwise healthy subjects has not been determined.

Therefore, a secondary analysis was performed of a large cohort of otherwise healthy African-American smokers and nonsmokers who participated in a genetic study of lung function and *F*eNO to determine the effect of age on *F*eNO

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Received:
July 12 2007
Accepted after revision:
September 17 2007

SUPPORT STATEMENT
The study was funded by the Sandler Program for Asthma Research (San Francisco, CA, USA), the National Institute of Environmental Health Sciences (ES011185; Research Triangle Park, NC, USA), and the National Center for Research Resources, Clinical Research Centers Program, National Institutes of Health (MO1-RR-30; Bethesda, MD, USA).

STATEMENT OF INTEREST
None declared.

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

levels. The current authors found that smoking-related declines in *F*eNO are greatest in older subjects. This suggests that smoking is associated with age-related declines in *F*eNO and justifies future mechanistic studies that address the impact of *F*eNO decline on lung function.

METHODS

Subject recruitment and enrolment

The present study results represent a secondary analysis of data obtained from subjects who were recruited as part of a study of the relationship between *F*eNO and type II inducible NO synthase (NOS2) genotypes present in African-Americans [33]. Healthy subjects aged 18–40 yrs were recruited from students and employees at local university campuses. In total, 994 subjects with adequate serum samples and *F*eNO levels were available for analysis. Informed consent was obtained as part of a protocol approved by the Duke University Institutional Review Board (Durham, NC, USA).

After giving informed consent, subjects were asked to provide their date of birth and declare that they were healthy (*i.e.* no chronic illnesses or chronic use of any medication except oral contraceptives), that they had no history of asthma, allergic rhinitis, hay fever or atopic dermatitis, that they were non-smokers, and that they were of African ancestry. Ethnicity was based on a self-declared description of ethnicity and determined using a questionnaire based on that developed by the US Census bureau. Blood samples for preparation of serum were obtained. *F*eNO was measured and a modified American Thoracic Society (ATS) questionnaire [34] was also administered to 524 subjects at the time of enrolment. The modified questionnaire collected information on smoking, cough, phlegm production, wheezing, dyspnoea and asthma history. The questionnaire also collected information about rhinitis, eczema and other allergy symptoms.

Measurement of FeNO

*F*eNO levels were measured in triplicate and averaged using a Sievers 280i Nitric Oxide Analyzer (NOA; GE Analytical Instruments, Boulder, CO, USA) according to the manufacturer's instructions. *F*eNO was measured according to ATS recommendations [35] at a flow rate of 50 $mL \cdot s^{-1}$ and against enough resistance to maintain an oropharyngeal pressure of $\geqslant 5$ cmH_20 and thereby permit closure of the soft palate and exclusion of nasal NO. Ambient air NO was excluded by inclusion of an activated charcoal and potassium hydroxide filter unit attached to the air intake of the NOA flow meter. Subjects were asked to breathe at least three times through the NOA flow meter with the attached activated charcoal and potassium hydroxide filter unit to reduce airway NO from ambient air prior to each *F*eNO measurement.

Serum total immunoglobulin E level measurements

Total serum immunoglobulin (Ig)E levels were measured using the Pharmacia CAP System (IgE FEIA; Pharmacia Diagnostics, Uppsala, Sweden).

Serum cotinine levels

Serum cotinine levels were determined using an ELISA-based assay from OraSure Technologies (Bethlehem, PA, USA). Manufacturer-specified cut-points for serum cotinine levels were used to separate subjects into smokers ($\geqslant 25$ $ng \cdot mL^{-1}$) and nonsmokers (<25 $ng \cdot mL^{-1}$).

Statistical analysis

Continuous variables (age, *F*eNO, total IgE, eosinophil cationic protein (ECP) and C-reactive protein (CRP) levels) were not normally distributed and were log normalised prior to statistical analysis. For the comparison of *F*eNO levels in subjects with different cotinine levels, ANOVA testing was used with multiple pairwise comparisons using Tukey–Kramer tests. For the linear regression analysis of cotinine levels and *F*eNO levels, cotinine levels were log normalised to permit a parametric analysis. For the analysis of current smokers and subjects that denied being ever smokers, cotinine levels were not log normalised because many cotinine levels were 0 $ng \cdot mL^{-1}$. In this analysis, cotinine levels were not normally distributed and nonparametric statistics (Wilcoxon rank-sum test) were used for this analysis. For univariate analyses of *F*eNO levels, unpaired t-tests were used for comparisons of smokers and nonsmokers. Subjects were dichotomised into younger (18–20 yrs) and older (21–40 yrs) groups based on the median age (20 yrs) of the cohort. Pearson's product–moment correlation coefficient was used for linear regression analyses of *F*eNO levels *versus* age and serum cotinine levels. Multiple linear regression was used for comparisons of *F*eNO levels while controlling for age, smoking status, sex, cotinine and total IgE levels. There were no corrections for multiple testing and the reported r^2 values were unadjusted.

RESULTS

Association of elevated cotinine levels with self-reported current cigarette smoking

The present authors' original *a priori* study of genetic correlates of *F*eNO levels was designed to exclude smokers. However, given the significant association of cigarette smoking with lower *F*eNO levels [1–8], ATS questionnaire data on smoking [34] were collected and serum cotinine levels were measured [36, 37] as a way to rigorously exclude smoking as a potential confounder of *F*eNO levels. To confirm the validity of using serum cotinine levels as a surrogate marker of cigarette smoking, cotinine levels were compared in subjects who self-reported cigarette smoking on the ATS questionnaire with subjects that did not report smoking. Of the 524 subjects who were administered ATS questionnaires, seven reported being current smokers and 471 denied being ever-smokers (*i.e.* subjects who reported being either current smokers or past smokers). Cotinine levels were higher in subjects who reported being current smokers *versus* subjects that denied being ever-smokers (median (interquartile range) 29.4 $ng \cdot mL^{-1}$ (0.3–130 $ng \cdot mL^{-1}$) *versus* 0.1 $ng \cdot mL^{-1}$ (0–0.6 $ng \cdot mL^{-1}$); $p=0.0025$, Wilcoxon rank-sum test). This analysis confirmed previous reports and validated the use of cotinine levels as a surrogate for cigarette smoking in the present cohort of subjects.

The manufacturer of the serum cotinine assay used in the present study recommends a cut-off of 25 $ng \cdot mL^{-1}$ for the identification of smokers. The current authors found that, despite self-reporting of nonsmoking by many subjects, 99 (10%) subjects had elevated serum cotinine levels ($\geqslant 25$ $ng \cdot mL^{-1}$) consistent with regular smoking. Given the inherent problems associated with self-reported information on smoking [38], the present authors elected to use serum cotinine levels as a surrogate for cigarette smoke exposure in the remainder of the analysis.

F_{ENO} in subjects with elevated serum cotinine levels

F_{ENO} levels were compared in subjects with serum cotinine levels <1 ng·mL^{-1} (subjects with minimal cigarette-smoke exposure), 1–25 ng·mL^{-1} (subjects with moderate cigarette-smoke exposure consistent with second hand-smoke exposure or occasional smoking) and ≥ 25 ng·mL^{-1} (subjects with high cigarette-smoke exposure and consistent with regular cigarette smoking). F_{ENO} levels were significantly lower only in subjects with serum cotinine levels ≥ 25 ng·mL^{-1} (fig. 1). Based on the results in figure 1, subjects were dichotomised into smokers and nonsmokers for all further analyses using a cotinine concentration of 25 ng·mL^{-1} as a cut-off for the two subject groups.

Consistent with prior studies [1–8], it was found that F_{ENO} levels were significantly lower in smokers compared with nonsmokers (geometric mean±SD): 13.9±18.0 ppb *versus* 20.5±21.3 ppb ($p<0.0001$); difference=6.6 ppb (confidence interval (CI) 4.1–9.2). As shown in figure 2, there was an association of F_{ENO} levels with serum cotinine levels in smokers; serum cotinine levels were inversely correlated with F_{ENO} levels in smokers ($r^2=0.13$; $p=0.0003$), suggesting that lower F_{ENO} levels were associated with increased levels of tobacco smoke exposure.

Age-related declines in F_{ENO} levels in smokers

The effect of age on F_{ENO} levels was examined in the present cohort. Consistent with prior studies [31], no correlation of age with F_{ENO} levels in nonsmokers was found (fig. 3a). In contrast, analysis of the relationship between F_{ENO} and age in smokers indicated that there was a significant decrease in F_{ENO} levels in older smokers (fig. 3b). A multiple linear regression model confirmed that age was significantly associated with F_{ENO} levels in smokers while controlling for cotinine levels (table 1).

In analyses stratified for age (based on the median age of the cohort, 20 yrs), comparison of F_{ENO} levels in younger subjects (18–20 yrs) did not reveal significant differences in F_{ENO} levels between smokers and nonsmokers, while F_{ENO} levels in older subjects (21–40 yrs) were significantly different between smokers and nonsmokers ($p<0.0001$; fig. 4). Among all subjects, the age-related difference in F_{ENO} levels between smokers and nonsmokers (*i.e.* the difference between 18–20-yr-old smokers and nonsmokers compared with the difference between 21–40-yr-old smokers and nonsmokers) was significantly different ($p<0.0001$; fig. 4) [39].

As there was an uneven distribution of subjects in each age group with regard to the variables listed in table 2, the present authors also tested whether smoking status was significantly associated with F_{ENO} levels in multiple linear regression models when subjects were stratified on the basis of age and while controlling for other variables (sex and total IgE levels; cotinine was not included because it is absent in most nonsmokers) associated with F_{ENO} levels [31, 40]. This analysis confirmed that smoking status was significantly associated with F_{ENO} levels in older subjects (aged 21–40 yrs, $p<0.0001$; table 3) but not younger subjects (aged 18–20 yrs, $p=0.16$; table 4). Likewise, when smoking status was included in a multiple linear regression model with age, sex and total IgE levels, there was a significant interaction of age and smoking status ($p=0.0008$, data not shown).

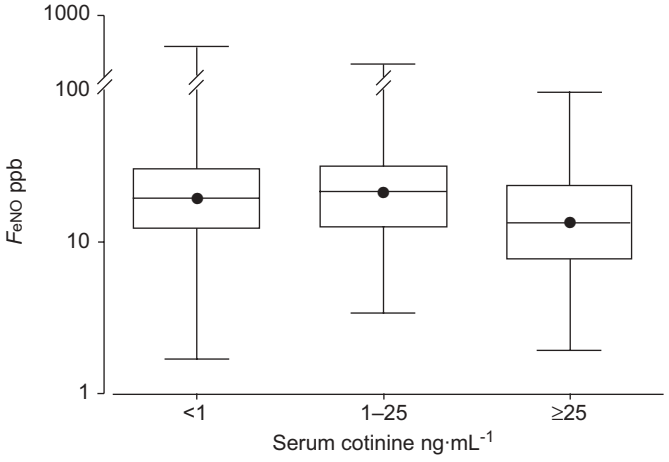

FIGURE 1. Box plot comparison of exhaled nitric oxide fraction (F_{ENO}) levels from subjects with serum cotinine levels <1 ng·mL^{-1} (n=770), 1–25 ng·mL^{-1} (n=126) and ≥ 25 ng·mL^{-1} (n=99). F_{ENO} levels were log normalised and compared using ANOVA ($p<0.0001$). Pairwise comparisons were performed with Tukey–Kramer tests and revealed significant differences between subjects with cotinine levels ≥ 25 ng·mL^{-1} and subjects with cotinine levels <1 ng·mL^{-1} ($p<0.0001$) and with cotinine levels between 1–25 ng·mL^{-1} ($p<0.0001$). A pairwise comparison between subjects with cotinine levels <1 ng·mL^{-1} and subjects with cotinine levels between 1–25 ng·mL^{-1} was not significant ($p>0.05$). The lower and upper limits of the rectangular box plots represent the 25th and 75th percentiles, respectively, for the data in each set. The upper and lower limits of the whisker represent the entire range of the dataset. The horizontal line in the centre of each box plot represents the median for that dataset. ●: geometric mean for each dataset.

DISCUSSION

The current authors believe that their study represents the largest analysis to date of the effect of smoking on F_{ENO} levels in otherwise healthy smokers. The study confirms previous

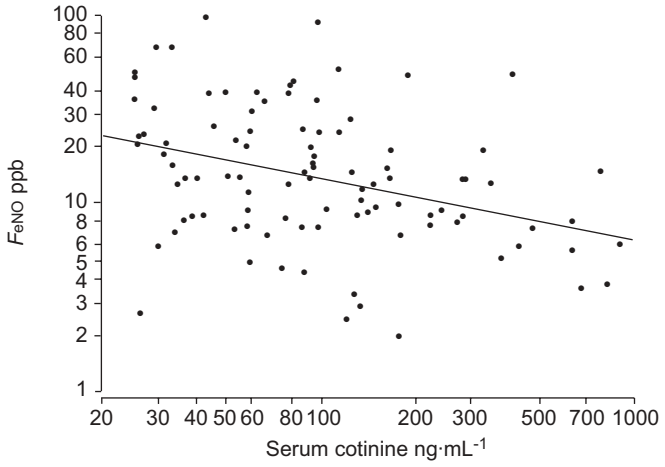

FIGURE 2. Plot of exhaled nitric oxide fraction (F_{ENO}) levels compared with serum cotinine levels in 99 smokers. F_{ENO} levels and serum cotinine levels were log normalised prior to analysis. Linear regression and Pearson's product–moment correlation coefficient were used to examine the relationship between F_{ENO} levels and serum cotinine levels. ——: derived from the linear regression analysis. $r^2=0.13$, $p=0.0003$.

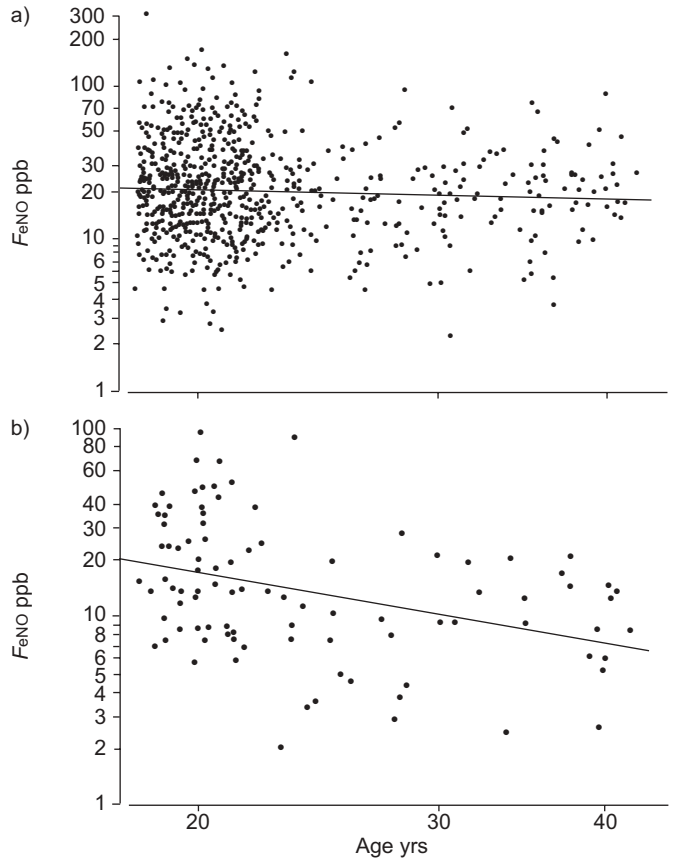

FIGURE 3. Plots of exhaled nitric oxide fraction (*F*eNO) levels compared with age in a) 98 African-American smokers and b) 876 African-American nonsmokers. *F*eNO levels and age were log normalised prior to analysis. Linear regression and Pearson's product–moment correlation coefficient were used to examine the relationship between *F*eNO levels and age. a) $r^2<0.01$, p=nonsignificant; b) $r^2=0.15$, p=0.0001; ——: derived from the linear regression analyses.

findings indicating that cigarette smoking was associated with lower *F*eNO levels [1–8] and that greater short-term (hours to days) exposure to inhaled tobacco smoke was associated with greater decreases in *F*eNO levels (fig. 2) [1]. The current study also addressed the hypothesis that smoking was associated with age-related declines in *F*eNO. Support for this hypothesis was demonstrated in two ways. First, an age-related decline in *F*eNO levels in smokers but not nonsmokers was demonstrated (fig. 3). Secondly, it was demonstrated that *F*eNO levels in older (21–40 yrs of age) smokers but not younger (18–20 yrs of age) smokers were significantly different from comparably aged nonsmokers (fig. 4).

Recent studies by McSharry *et al.* [32] and Malinovschi *et al.* [10] did not observe a similar age-related decline in *F*eNO levels in healthy nonasthmatic smokers. The current authors believe that the present results were different from the results of the other two other studies because they had smaller sample sizes and did not include subjects <21 yrs of age. For example, had the present study only analysed the correlation between age and *F*eNO in the subset of 54 smokers in the cohort aged >21 yrs, a correlation between age and *F*eNO levels would not have been observed ($r^2=0.0223$, p=0.2809). However, given the limited numbers of subjects in the current study who were in their late 30's, it remains possible that the analysis in figure 3a was unduly affected by low *F*eNO levels in some of the oldest smokers in the cohort.

TABLE 1 Multiple linear regression analysis of factors associated with exhaled nitric oxide fraction (*F*eNO) levels in 98 healthy African-American smokers[#]

Factor	Effect	Estimate	r^2	p-value
Age yrs[¶]	Inverse	-1.0166	0.1465	0.0013
Serum cotinine level	Inverse	-0.2676	0.2262[+]	0.0023

[#]: in contrast to nonsmokers, total immunoglobulin (Ig)E levels and sex in smokers were not significantly associated with *F*eNO levels after adjustment for age and serum cotinine levels (data not shown). Eosinophil cationic protein levels are co-linear with IgE levels (data not shown). IgE levels had a stronger relationship with *F*eNO levels and were tested in the model instead of eosinophil cationic protein levels. C-reactive protein levels were not associated with *F*eNO levels and were not included in the analysis (data not shown). p<0.0001 for *F*eNO ANOVA using age and serum cotinine level. [¶]: all continuous variables were log normalised prior to analysis. There was no significant interaction between age and cotinine levels (data not shown). [+]: cumulative r^2 value, *i.e.* r^2 value for serum cotinine level, includes effect of age and serum cotinine levels on variability of *F*eNO levels.

Like the studies of McSharry *et al.* [32] and Malinovschi *et al.* [10], other studies of smoking and *F*eNO have not included

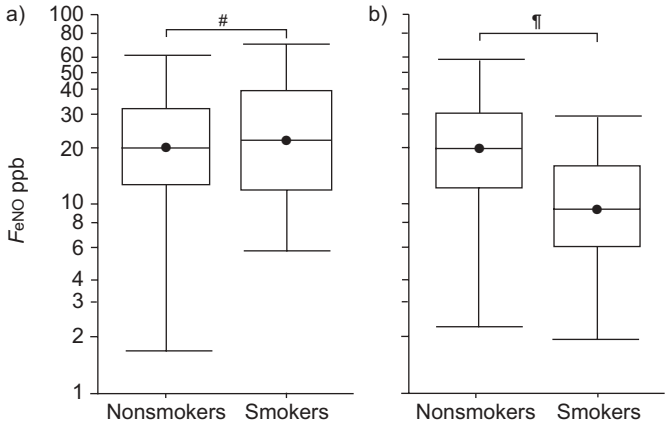

FIGURE 4. Box plot comparison of exhaled nitric oxide fraction (*F*eNO) levels from African-American subjects with serum cotinine levels <25 ng·mL^{-1} (non-smokers) and ⩾25 ng·mL^{-1} (smokers) in a) subjects aged 18–20 yrs (515 and 44 subjects, respectively) and b) subjects aged 21–40 yrs (363 and 54 subjects, respectively). *F*eNO levels were log normalised and compared using an unpaired t-test (comparison of *F*eNO levels for nonsmokers *versus* smokers was p=0.9404 and p<0.0001 in a and b, respectively). The age-related difference in *F*eNO levels between smokers and nonsmokers was p<0.0001. The lower and upper limits of the box plots represent the 25th and 75th percentiles, respectively, for the data in each set. The upper and lower limits of the whisker plots represent the upper and lower quartiles ±1.5 times the interquartile range, respectively, in each dataset. The horizontal line in the centre of each box plot represents the median for that dataset. ●: geometric mean for each dataset. [#]: p=nonsignificant; [¶]: p<0.0001.

TABLE 2 Demographic characteristics and serum concentrations of inflammatory markers and cotinine levels of subjects[#]

Factor	All	Age yrs		p-value[¶]
		18–20	21–40	
Sex female/male	651 (66)/338 (34)	372 (68)/179 (32)	269 (64)/152 (36)	0.2461
Nonsmoker/smoker	900 (90)/99 (10)	518 (92)/44 (8)	365 (87)/54 (13)	0.0098
Serum total IgE kU·L^{-1}	61±200	67±230	57±170	0.0821
Serum cotinine ng·mL^{-1}[+]	97±140	82±120	110±160	0.0717

Data are presented as n (%) or geometric mean±SD. Complete information on all subjects was not available. The numbers in the chart represent the numbers of subjects with each characteristic where complete information was available. Dichotomous variables were compared using two-tailed Fisher's exact test. Continuous variables were log normalised and compared using unpaired t-tests. Ig: immunoglobulin. [#]: in total, 994 subjects were enrolled in the study; [¶]: comparisons between 18–20-yr-olds and 21–40-yr-olds; [+]: among subjects with serum cotinine concentrations ⩾25 ng·mL^{-1}.

subjects aged <21 yrs and, therefore, would not have reached the same conclusions as the present study regarding *F*eNO levels in young smokers compared with nonsmokers [1–4, 6]. One possible explanation for the current findings regarding the lack of a difference in *F*eNO levels in young smokers compared with nonsmokers is related to the known association of increasing age in children (but not adults) with increasing *F*eNO [40–42]. As such, continued increases in *F*eNO in adults <21 yrs of age may have counteracted smoking-related declines in *F*eNO in this age group and accounted for the findings regarding the lack of a difference in *F*eNO levels in young smokers compared with nonsmokers.

Long-term smoking leads to functional and structural changes in the lung. The present results on the age-related decline in *F*eNO suggest that the duration of tobacco-smoke exposure might be associated with decreased *F*eNO levels. The current data does not include self-reported information on the length of time that subjects smoked, although >80% of adult smokers start smoking before the age of 18 yrs [43], suggesting that subject age is a reasonable correlate of duration of tobacco-smoke exposure. While other factors besides smoking may account for the age-related decline in *F*eNO levels in smokers, the present authors believe this is unlikely because there were no age-related changes in *F*eNO levels in nonsmokers (fig. 3a).

TABLE 3 Multiple linear regression analysis of factors associated with exhaled nitric oxide fraction (*F*eNO) levels in 417 healthy African-American smokers and nonsmokers aged 21–40 yrs[#]

Factor	Effect	Estimate	r^2	p-value
Smoking status	NS>S	0.17448	0.0994	<0.0001
Total IgE level[¶]	Direct	0.12644	0.1594	<0.0001
Sex	M>F	0.07355	0.2073[+]	<0.0001

Ig: immunoglobulin; NS: nonsmoker; S: smoker; M: male; F: female. [#]: p<0.0001 for *F*eNO ANOVA using smoking status, sex and total IgE levels; [¶]: all continuous variables were log normalised prior to analysis; [+]: cumulative r^2 value, *i.e.* r^2 value for sex, includes effect of smoking status, total IgE and sex on variability of *F*eNO levels.

The presence of mild chronic obstructive pulmonary disease (COPD) in older subjects may have influenced the results. Although Roy *et al.* [44] recently found that COPD was associated with decreased airway NO levels, most studies suggest that COPD is associated with increased *F*eNO levels [4, 45] and, if anything, this would have diminished the difference in *F*eNO levels between older smokers and nonsmokers. Taken together, the current authors believe that long-term smoking may result in permanent reductions in *F*eNO levels, possibly *via* airway epithelial lining changes and changes in NOS2 expression and NO production.

Tobacco-smoke exposure is associated with airway epithelial hyperplasia, mucus cell hyperplasia, airway metaplasia, epithelial damage and airway neutrophil infiltration [16, 17]. Many of these changes persist after subjects quit smoking, including persistence of neutrophil inflammation [16, 17]. The hypothesis that long-term cigarette smoking is associated with permanent reductions in *F*eNO is supported by studies on older smokers who quit smoking [2, 6]. While these studies indicated that *F*eNO levels increased in subjects after they quit smoking, former smokers still had lower *F*eNO levels compared with healthy control subjects [2, 6]. Likewise, Malinovschi *et al.* [10] found that ex-smokers had significantly lower *F*eNO levels than never-smokers. A prospective, longitudinal study of abstaining smokers who had previously smoked cigarettes for various

TABLE 4 Multiple linear regression analysis of factors associated with exhaled nitric oxide fraction (*F*eNO) levels in 559 healthy African-American smokers and nonsmokers aged 18–20 yrs[#]

Factor	Effect	Estimate	r^2	p-value
Total IgE level	Direct	0.16198	0.1231	p<0.0001
Sex	M>F	0.07143	0.1662[¶]	p<0.0001

All continuous variables were log normalised prior to analysis. When added to the model, the p-value for smoking status as an individual variable was 0.1654. Ig: immunoglobulin; M: male; F: female. [#]: p<0.0001 for *F*eNO ANOVA using sex and total IgE levels; [¶]: cumulative r^2 value, *i.e.* r^2 value for sex, includes effect of total IgE levels and sex on variability of *F*eNO levels.

durations would provide better evidence to support the hypothesis that long-term smoking may be associated with permanent reductions in *F*eNO levels.

The design of the present study did not allow direct examination of respiratory epithelium in smokers or the correlation of epithelial damage with *F*eNO. Therefore, other hypotheses that account for the age-related decline in *F*eNO in smokers are also possible. Smoking-related downregulation of epithelial NOS2 enzyme activity and smoking-related increases in airway enzymes and reactive oxygen species that react with or metabolise NO are possible aetiologies for the age-related decline in *F*eNO in smokers [10, 46]. Another hypothesis relates smoking-induced pulmonary changes due to COPD as the cause of decreased *F*eNO in smokers. As stated earlier, while most studies found that *F*eNO levels increase in patients with COPD [4, 45], more recent data from ROY *et al.* [44], using multiple measurements of exhaled NO at different flow rates to model airway NO, suggests that *F*eNO levels are lower in COPD patients. Like the hypothesis about smoking-related respiratory epithelial damage, the COPD hypothesis would associate COPD-related inflammatory changes and parenchymal lung damage with reductions in *F*eNO. Although the current results do not provide definitive evidence to address these hypotheses as the cause of decreased *F*eNO levels in smokers, the results of the present study clearly support an association of an age-related decline in *F*eNO in smokers.

While the present authors focused on the possibility that smoking has significant age-related effects on airway respiratory epithelium, it is possible that other compartments within the lung may contribute to the lower *F*eNO levels associated with smoking. All three isoforms of NOS are expressed in the lung [11]. Techniques that permit separate measurement of the airway and alveolar components of *F*eNO would be useful in this context to address the hypothesis that smoking primarily alters airway respiratory epithelial production of *F*eNO [47]. Experiments using intravenous and inhaled inhibitors of the different isoforms of NOS would also be useful to delineate the relative contributions of neural NOS1, airway NOS2 and vascular NOS3 to the difference in *F*eNO between older smokers and nonsmokers [15, 48].

Measurement of serum cotinine, a nicotine metabolite, is an objective surrogate for quantifying tobacco-smoke exposure [49]. Cotinine levels can be accurately measured in blood, urine, saliva and other body fluids. The half-life of cotinine in serum is 15–17 h, which allows for accurate estimation of daily tobacco-smoke exposure in randomly obtained blood specimens [49]. Serum cotinine levels correlate well with the number of cigarettes smoked and with the amount of time since a cigarette was smoked [50–52]. Information on the number of cigarettes that subjects smoked was not collected in the current study and, as such, relied on the use of serum cotinine levels as a surrogate marker of cigarette consumption.

The present authors believe that serum cotinine levels represent the best available assessment of smoking status in the cohort as subject self-reports of smoking habits did not adequately exclude smokers. Based on data provided by the manufacturer of the cotinine assay and published studies [53], it is believed that the manufacturer-recommended cut-off of 25 ng·mL^{-1} for serum cotinine levels is conservative and unlikely to include many nonsmoking subjects exposed to passive smoke. The significantly lower *F*eNO levels in subjects with serum cotinine levels $\geqslant$25 ng·mL^{-1} (fig. 2) and the comparable *F*eNO levels in current smokers *versus* subjects with serum cotinine levels $\geqslant$25 ng·mL^{-1} also supports the rationale for selecting 25 ng·mL^{1} as a cut-off for classification of smoking status. The current data did not indicate lower *F*eNO levels in subjects with cotinine levels between 1–25 ng·mL^{-1} and there was only a weak correlation between *F*eNO and serum cotinine levels in subjects with cotinine levels between 1–25 ng·mL^{-1} (r^2=0.03, p=0.0410). Furthermore, serum cotinine concentrations >1 ng·mL^{-1} and <25 ng·mL^{-1} are consistent with exposure to second-hand smoke [53]. In contrast to the declines in *F*eNO associated with cigarette smoking, a recent publication found that exposure to secondhand smoke was associated with modestly increased *F*eNO levels [53]. Taken together with the present data on the relationship of *F*eNO levels and serum cotinine levels (fig. 2), the current authors believe that this data supports dichotimisation of subjects into smokers and nonsmokers on the basis of a serum cotinine level of 25 ng·mL^{-1}.

In summary, the present results indicated that smoking had different impacts on exhaled nitric oxide levels in subgroups of otherwise healthy smokers. Smoking-related declines in exhaled nitric oxide levels appeared to be disproportionately greater in older individuals. This suggested that smoking was associated with age-related declines in exhaled nitric oxide and justifies future mechanistic studies that address the impact of exhaled nitric oxide fraction decline on lung function.

ACKNOWLEDGEMENTS

The authors are grateful for the assistance of E. Scarlett and C. Foss during subject enrolment and sample collection. They also acknowledge C. Thomas and C. Tao for assistance with computer data entry. The use of J.B. Weinberg's nitric oxide analyser and W. Burks' (all affiliated with Duke University Medical Center, Durham, NC, USA) CAP-FEIA instrument for measurement of exhaled nitric oxide levels and total IgE levels, respectively, was greatly appreciated.

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