

PATHOGENESIS

Predictors of neutrophilic airway inflammation in young smokers with asthmaChristian Grabow Westergaard¹, Christian Munck², Jens Helby³, Celeste Porsbjerg¹, Lars H. Hansen⁴, and Vibeke Backer¹¹Respiratory Research Unit, Bispebjerg University Hospital, ²Department of Systems Biology, Technical University of Denmark, ³Department of Clinical Microbiology, Rigshospitalet, University Hospital of Copenhagen, and ⁴Department of Biology, University of Copenhagen, Copenhagen, Denmark**Abstract**

Introduction: Asthma is one of the most widespread chronic diseases worldwide. In spite of numerous detrimental effects on asthma, smoking is common among asthma patients. These smoking-induced aggravations of asthma may be attributed to changes in airway inflammation, which is characterized by a higher degree of neutrophilic inflammation than in non-smokers. A state of neutrophilic inflammation may lead to increased steroid resistance and an accelerated loss of lung function owing to tissue destruction. The aim of this study was to elucidate predictors of neutrophilic inflammation in young asthmatic smokers not on steroid treatment, including analysis of tobacco history and bacterial colonization. **Methods:** In a cross-sectional study, 52 steroid-free, current smokers with asthma were examined with induced sputum, fractional exhaled nitric oxide (FeNO), lung function, ACQ6 score, mannitol and methacholine challenge. A sample from the sputum induction was taken for bacterial analysis using 16S gene PCR technique and sequencing. **Results:** Using one-way analysis of variance and binary and linear regression models, only age and ACQ6 score were found to be significant predictors for airway neutrophilia. The investigation also included analysis for effect of pack years, current tobacco consumption, body mass index, lung function, FeNO; methacholine and mannitol responsiveness, atopy, gender, asthma history and presence of bacteria. The most common potentially pathogenic bacteria found were *Streptococcus spp.*, *Haemophilus spp.* and *Mycoplasma spp.* **Conclusion:** In this study, no tobacco-related predictors of airway neutrophilia were found, indicating that in the younger years of asthma patients who smoke, the amount of tobacco smoked in life does not influence the degree of neutrophilia. Conversely, for asthmatic smokers, neutrophilia may be induced when a certain threshold of tobacco consumption is reached.

Keywords

Biomarkers, mechanisms, pathophysiology, tissue damage, tobacco abuse

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Introduction

Asthma is a chronic disease affecting the airways of more than 300 million people worldwide [1], and it is characterized by respiratory symptoms such as wheezing breath, dyspnoea, coughing and chest tightness due to airway inflammation and airway hyperresponsiveness (AHR). Asthma is commonly triggered not only by specific agents like aeroallergens or by physical exercise but also non-specifically by air pollution and smoke.

A substantial challenge in asthma management is the large proportion of asthmatic smokers. In the western world, cigarette smoking is as common among asthma patients as in the population in general [2]. This is very unfortunate and surprising considering the fact that asthmatic smokers have to struggle with a higher degree of symptoms, attenuated effect

of asthma medication [3] and more frequent exacerbations and hospital admissions than do the non-smoking asthmatics [2]. The risk of asthma-caused death is also higher among the smokers [2]. Furthermore, owing to chronic asthmatic airway inflammation and exposure to tobacco smoke, the decline in lung function with age is significantly accelerated in asthmatic smokers, in some cases, leading to early development of COPD [4–7].

These detrimental effects of tobacco smoke on most clinical asthma parameters can, in part, be attributed to changes in airway inflammation. Thus, it has previously been demonstrated that the sputum of smokers contains an increased number of neutrophils compared to non-smokers [8,9].

The inflammatory changes in asthmatic smokers show some resemblance to the inflammatory findings among most patients with COPD. In a small study, it was shown that smokers with asthma expressed structural changes similar to those found in early COPD, such as reduced diffusion capacity, FEV₁/FVC ratio and FRC [9]. It is possible that

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these changes are associated with increased neutrophilia, leading to enhanced activity of neutrophil elastase, an enzyme contributing to tissue destruction. As a rule, the pathological changes in patients with COPD develop gradually after many years of tobacco exposure. However, in asthma patients, it is unknown how much tobacco consumption (dose) is required or when the smoking-induced inflammatory changes start to occur. In this study, we hypothesized that the neutrophils in the sputum of young asthma patients would be correlated to the number of pack years.

Other factors than tobacco smoking, such as air pollution [10], obesity [10], occupational agents [10], increasing age [11] and steroid treatment [12], have been shown to predispose for airway neutrophilia in asthma. Furthermore, it has been demonstrated that asthma patients are colonized with a higher number of *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* than healthy controls [13], and that airway neutrophilia in asthma can be mitigated using macrolide antibiotics [14]. In asthmatic smokers, it is not known whether chronic colonization or infection with these or other bacteria are causes of heightened neutrophilic inflammation due to oxidative stress and thereby increased interleukin 8 production owing to the persisting presence of bacteria in the airway epithelium.

The aim of this study was to elucidate different factors potentially contributing to airway neutrophilia in young asthmatic smokers not on steroid treatment, focusing especially on life tobacco consumption and presence of airway bacteria.

Materials and methods

Design

This was a cross-sectional study of young asthma patients, currently smoking and not treated with inhaled steroids. The patients were recruited consecutively, mostly through newspaper advertisements or through posters in waiting rooms of general practitioners in the Copenhagen area. All subjects had the same screening procedure performed and were included on the basis of current asthma symptoms and an objective positive diagnostic test for asthma.

Materials

Study group

Steroid-free smoking asthma patients were included, aged 18–40 years. Fifty-two asthmatic smokers met the inclusion criteria and were included in this study. The inclusion criteria were as follows: (1) current smoking (verified with CO breath test) with a daily consumption of at least 10 cigarettes per day within the past year and a total consumption of at least 10 pack years, (2) current asthma symptoms within the past year, confirmed through interview with study physician and (3) at least one positive objective diagnostic test suggestive of asthma: positive methacholine test ($PD_{20} < 8 \mu\text{mol}$), positive mannitol test ($PD_{15} < 635 \text{ mg}$), FEV_1 reversibility to β_2 -agonist of $\geq 12\%$ and 250 ml, or a two-week day-to-day FEV_1 variation of $\geq 20\%$. The exclusion criteria were as follows: (1) treatment with ICS, oral steroids, leukotriene antagonist, long-acting β_2 -agonists or anticholinergics within 3 months

prior to inclusion, (2) lower respiratory tract infections within 3 months, (3) a medical history of other chronic lung disease, including COPD and (4) pregnancy.

Methods

After participants had signed an informed consent form, the screening procedure was performed. This comprised clinical interview with asthma classification and recording of tobacco history along with current consumption, followed by skin prick test, lung function testing and a methacholine challenge. Furthermore, a 14-day FEV_1 home measurement was commenced. At a later date, a mannitol challenge was performed, but only in patients with a FEV_1 of at least 60% of expected. Subsequently, on a third day, the baseline visit was performed; this included measurement of lung function, a bronchial challenge with methacholine, inflammatory tests (fractional exhaled nitric oxide, $FeNO$, and induced sputum), CO measurement and ACQ score. Data from this baseline visit were used in the statistical analysis, in addition to the data regarding atopy and tobacco history from the screening visit.

Descriptions of the tests used

- (1) Spirometry (EasyOne™, ndd Medizintechnik AG, Zurich, Switzerland): best of three measurements of FEV_1 , FVC and FEV_1/FVC ratio in accordance with ATS recommendations [15]. Reference values according to fixed data of the spirometer. Reversibility test 20 minutes after inhalation with terbutaline sulfate (Bricanyl Turbuhaler) 1.5 mg.
- (2) $FeNO$ (NIOX, Intra Medic, Copenhagen, Denmark): average of three measurements (flow rate 50 ml) was measured in accordance with the ATS/ERS guidelines [16].
- (3) Mannitol provocation test (Osmohale™ Mannitol, Pharmaxis, Buckinghamshire, UK): inhalation of ascending dosages 0, 5, 10, 20, 40, 80, 160, 160, 160 mg [17]. A positive test was defined as a 15% fall in FEV_1 or more and resulted in termination of test. RDR was calculated as: $(100 \times (\text{initial } FEV_1 - \text{last dose } FEV_1) / \text{initial } FEV_1) / \text{cumulated dose of mannitol in mg}$. If FEV_1 was $< 60\%$ of expected, mannitol challenge was not performed.
- (4) Methacholine provocation test with the Yan method (Nebicheck device, nebulized fluid: methacholine bromide) [18]: inhalation of ascending dosages, cumulated: 0, 0.03, 0.06, 0.12, 0.25, 0.50, 1.00, 2.00, 4.00, 8.00 μmol . Measurements of FEV_1 exactly one minute after inhalation. A 20% fall in FEV_1 or more was defined as a positive result and terminated the test.
- (5) Induced sputum: sputum preparations were collected in accordance with published guidelines [19]. Samples were fixed with methanol and dyed with eosin Y 2% (BioStain, Manchester, UK), methylene blue $< 2\%$ and azur I $< 2\%$ (BioStain). Dyed slides were counted by the study physician with a Zeiss Axio microscope. Four hundred non-squamous cells (eosinophils, neutrophils, macrophages, lymphocytes and cylindrical epithelial cells) were counted for each patient in order to determine cell distribution. Sputum neutrophils/

eosinophils cells per gram sputum were calculated as: $10^6 \times (\text{neutrophil or eosinophil-}\%/100) \times (((\text{living cell count} + \text{dead cell count}) \times 2 \times (\text{filtered sputum mass}))/(\text{no. of grid cells counted})/100)/(\text{sputum mass in g})$. Phenotyping was done in accordance with previous definitions [20]. IL-8 and neutrophilic elastase were measured in sputum supernatant using enzyme-linked immuno sorbent assay kits (Quantikine® ELISA, R&D systems and InnoZyme TM Human Neutrophil Elastase Immunocapture Activity Assay Kit, Calciocem®) in accordance with manufacturer guidelines. Using sputum material from a sputum sample picked up under sterile conditions, up to 1 ml of each sputum sample was transferred to a tube containing 4 ml 27% glycerol in lysogeny broth and frozen immediately. These frozen sputum/glycerol samples were used for the bacteriological analysis.

- (6) Skin prick test: a standard panel of common allergens was used: cat, dog, horse, Alternaria, Cladosporium, birch, mugwort, grass, *Dermatophyoides pteronyssinus* and *Dermatophyoides farinae*. Atopy was defined as at least one positive response with a wheal diameter of at least 3 mm.
- (7) Home FEV₁ measurements: Piko-6 (Ferraris, UK) used twice daily for two weeks. Day-to-day FEV₁ variation was calculated as follows: $((\text{highest measured FEV}_1 - \text{lowest measured FEV}_1)/\text{highest measured FEV}_1) \times 100$.
- (8) CO breath test: Bedfont Micro⁺ Smokerlyzer or CO monitor, Bedfont Scientific Ltd., Kent, UK. Cut-off value was 10 ppm.
- (9) Questionnaires: Asthma control questionnaire (ACQ) [21]. ACQ6 without lung function measurement was applied.
- (10) Bacterial analysis: total DNA was extracted from the frozen sputum/glycerol samples using the PowerLyzer™ PowerSoil® DNA Isolation Kit (MOBIO, Carlsbad, CA). The 16S gene was amplified using the 341F primer CCTAYGGGRBGCASCAG and the 806R primer GGA CTACNNGGTATCTAAT. Each 16S library was barcoded and sequenced using the 454 FLX platform (Roche, Branford, CT). The sequence data was analyzed using the QIIME pipeline version 1.6.0 (Boulder, CO) [22]. Operational taxonomic units (OTUs) were defined using an identity cut-off of 97%, and taxonomy was assigned with the RDP classifier. The OTU table was mined for the pre-defined set of airway pathogens using R [23]. For each patient, the total number of 16S sequences belonging to each of three potentially pathogenic bacteria (*Streptococcus*-, *Haemophilus*- and *Mycoplasma* spp.) were extracted. To analyze the proportion of potentially pathogenic bacteria relative to the total number of bacteria in each patient, we calculated the percentage of sequences from each of the three pathogens relative to the total 16S sequence count.

Statistics

All statistical analysis was performed with IBM SPSS Statistics version 20 (SPSS Inc., Chicago, IL). Chi-squared

analysis was used for crosstabs. For parametric independent two-sample test, unpaired *t*-test was used. If possible and if necessary, logarithmic transformation was performed before *t*-test analysis. Mann–Whitney U test was used for non-parametric two-sample test. Pearson's *r* or Kendall's tau was used for correlation analysis. Due to two negative mannitol RDR values and one negative methacholine RDR value, values of 0.008 and 0.5 were added to all mannitol and methacholine RDR values, respectively, in order to allow for logarithmic transformation. Subsequently, the same values were subtracted after calculating means and CI. Significance level was $p \leq 0.05$.

Analysis for possible biasing scale variables on the primary outcome variables (pack years, BMI, age, FEV₁-% of predicted, FEV₁/FVC% index, FVC, asthma years of duration, methacholine RDR, mannitol RDR and ACQ6 score) was performed using one-way analysis of variance (ANOVA) with the presence of neutrophilia as factor variable. For possible biasing categorical variables (current daily tobacco use, gender and atopy), χ^2 test was used.

Variables with $p < 0.50$ from the one-way ANOVA were entered in three linear regression models with log transformed neutrophils per gram sputum, neutrophil % as well as IL-8 as dependent variables. The backward method was applied. In addition, a similar binary logistic regression model was applied using the same possible predictor variables and the presence of neutrophilia (cut-off 55%) as dependent variable. In this model, Wald statistics was used. The study was approved by the local ethical committee (no. H-2-2010-140). All participants signed an informed consent.

Results

In total, 76 asthmatic smokers completed asthma screening. Fifty-two met all requirements and were included in this study. Of these 52 patients, 44 subjects completed all three visits, eight did not perform the mannitol test.

On an average, the patients had been diagnosed with asthma for more than 10 years (Table 1), whereas 26% of the subjects were diagnosed with asthma during the study process (not shown). The mean FEV₁% of predicted was lower than expected in a normal population (84.3%). The mean BMI of the patients was within normal range (25.4). The patients

Table 1. Baseline characteristics expressed as mean $\pm$ SD.

Number of subjects	52
Females	42%
Age (years)	31.4 $\pm$ 5.9
No. of pack years	15.4 $\pm$ 4.3
Asthma, years since time of diagnosis	14.9 $\pm$ 12.6
Current daily tobacco consumption	
10–20 cigarettes	55%
21–30 cigarettes	34%
>30 cigarettes	11%
Atopy	55%
FEV ₁ % of expected	84.3 $\pm$ 15.8
FVC % of expected	98.0 $\pm$ 14.0
FEV ₁ /FVC %	73.0 $\pm$ 10.4
Body mass index (kg/m ²)	25.4 $\pm$ 4.1
ACQ6 score	1.7 $\pm$ 0.8
No. of puffs rescue medication per day	0.9 $\pm$ 1.2
FeNO (ppb) (GM (CI))	8.0 (6.2–10.2)

were symptomatic with a mean ACQ score of 1.7, but had a low usage of short-acting bronchodilators (0.7 times daily). Most patients smoked between 10 and 20 cigarettes per day, and only 11% smoked more than 30. In five patients, current tobacco consumption was not recorded.

AHR to the bronchial provocations with methacholine and mannitol was frequently found to be 75% and 70%, respectively (Table 2). Among the 44 patients, who had both asthma challenges performed, 24 were positive to both tests.

Inflammatory cells and biomarkers in sputum

The geometric mean (GM) proportions of eosinophils and neutrophils in the patients were 0.89% and 54.2%, respectively (Table 3). The sputum samples of the groups contained 11.7% squamous cells (degree of pollution). Significant correlations were found between both IL-8 and neutrophilic elastase and neutrophilic-% and neutrophils per gram sputum (Table 4). The four sub-phenotypes were almost equally distributed with the eosinophilic and the paucigranulocytic sub-phenotypes as the most frequent (29%) (Table 3). Neutrophilia (neutrophilic or mixed granulocytic phenotypes) was present in 42% of the smokers.

Table 2. AHR variables.

No. of methacholine-positive subjects	75%
Methacholine RDR (% FEV ₁ fall per µmol) (GM (CI))	8.8 (5.6–13.7)
No. of mannitol-positive subjects of 44 subjects	70%
Mannitol RDR (% FEV ₁ fall per mg) (GM (CI))	0.041 (0.027–0.059)

Table 3. Inflammatory characteristics in induced sputum expressed as mean ± SD.

Sputum eosinophil-% (GM (CI))	0.89 (0.51–1.57)
Sputum neutrophil-%	54.2 ± 19.5
Sputum lymphocytes-% (median (min–max))	2.3 (0.0–47.5)
Sputum columnar cell-% (median (min–max))	1.3 (0.0–17.0)
Sputum macrophage cell-%	34.1 ± 17.6
Sputum pollution-% (GM (CI))	11.7 (9.1–15.1)
Neutrophil cells per gram sputum × 10 ⁶ (GM (CI))	1.21 (0.95–1.56)
Eosinophil cells per gram sputum × 10 ⁶ (GM (CI))	0.022 (0.012–0.039)
Sputum IL-8 pg/ml (GM (CI))	525.5 (415.0–665.5)
Sputum neutrophilic elastase ng/ml (median (min–max))	17.2 (0–83.9)
Eosinophilic	15 (29%)
Mixed granulocytic	13 (25%)
Neutrophilic	9 (17%)
Paucigranulocytic	15 (29%)

Distribution of inflammatory phenotypes according to Simpson et al. [20].

Characteristics of patients grouped according to neutrophilia

When grouping patients into a neutrophil and a non-neutrophil group with a 55% cut-off value, only ACQ6 score was significantly different between the groups (1.3 vs. 1.9, $p = 0.008$), highest in the group of neutrophil >55%. No other of the predictor variables were significantly different between the groups (Table 5). Only gender showed a difference with a significance level of less than 0.20 (36% females versus 57% males, $p = 0.148$).

Streptococcus spp. and *Haemophilus* spp. each constituted about 4–6% (GM) of the bacteria found in the sputum, regardless of the presence of sputum neutrophilia. *Mycoplasma* spp. was far less frequently found.

The following variables were found somewhat different between the groups ($p < 0.50$) and were entered in the regression models as predictors: ACQ6, age, atopy, BMI, methacholine RDR, FeNO, *Mycoplasma* spp. and *Streptococcus* spp. (Table 5). In addition, pack year number was included in the analysis. FEV₁-%, FVC-%, asthma years of duration, *Haemophilus* spp. and current tobacco consumption were found to be unassociated with increased neutrophils.

Regression models

In a multiple linear regression analysis using sputum neutrophil-% as dependent variable, age, pack years and ACQ6 score was found to be significant predictors but pack years with a negative B value (Table 6). FeNO was a near-significant negative predictor ($p = 0.070$), whereas gender, atopy, BMI, *Mycoplasma* spp. and *Streptococcus* spp. were of no significant importance as predictors for having neutrophilic sputum. Using log transformed neutrophils per gram sputum in a multiple linear regression, no significant predictors were found. Using IL-8 as a biomarker for neutrophilic inflammation, in a multiple linear regression as dependent variable and continuous predictor for neutrophilic sputum, age was a near-significant predictor ($p = 0.070$). No other significant predictors were found.

In the binary logistic regression model using a cut-off value of 55% (this value was chosen as opposed to 61% in order to increase sample size in the high-neutrophilia group), with neutrophilia as a dichotomous-dependent variable and including the same covariates as in the linear regression, age ($p = 0.038$) and ACQ6 ($p = 0.004$) were found to be significant predictors. No other significant predictors were found.

Discussion

In this study of 52 young, steroid-free asthmatic smokers, we were not able to demonstrate any association between the cumulated or current consumption of tobacco and the

Table 4. Correlation coefficients between neutrophils and biomarkers in sputum.

	Sputum neutrophil-%	Sputum neutrophil cells per gram sputum	Sputum IL-8
Sputum IL-8	0.227 ($p < 0.001$)	0.339 ($p < 0.001$)	–
Sputum neutrophilic elastase	0.293 ($p = 0.002$)	0.491 ($p < 0.001$)	0.291 ($p = 0.002$)

Sputum IL-8 was analyzed with Pearson's r and sputum neutrophilic elastase with Kendall's τ .

Table 5. Variables of patients with and without sputum neutrophilia expressed in mean $\pm$ SD.

	Sputum neutrophils <55% (N = 25)	Sputum neutrophils >55% (N = 27)	p
Females %	32%	52%	0.148
Pack years	15.2 $\pm$ 4.1	15.6 $\pm$ 4.6	0.747
Current tobacco consumption (cigarettes per day)			
10–20	55%	56%	0.813
21–30	32%	36%	
>30	13%	8%	
Age, years	30.6 $\pm$ 5.9	32.1 $\pm$ 5.9	0.360
Asthma duration, years	14.7 $\pm$ 13.1	15.1 $\pm$ 12.4	0.899
FEV1 % expected	84.9 $\pm$ 12.1	83.7 $\pm$ 18.8	0.791
FVC % expected	98.9 $\pm$ 13.7	97.2 $\pm$ 14.5	0.674
Home FEV1 % variation	31.0 $\pm$ 16.9	29.2 $\pm$ 12.7	0.735
Body mass index (kg/m ²)	25.0 $\pm$ 3.9	25.8 $\pm$ 4.3	0.471
Atopy	63%	48%	0.304
ACQ6 score	1.3 $\pm$ 0.7	1.9 $\pm$ 0.9	0.008
No. of puffs rescue medication per day	0.9 $\pm$ 1.1	0.9 $\pm$ 1.3	0.874
β 2-agonist reversibility, % (median (min to max))	–1.1 (–7.7 to +32.4)	1.4 (–10.5 to +20.7)	0.776
Methacholine RDR (GM (CI))	9.5 (5.7–15.7)	6.9 (3.2–14.1)	0.463
Mannitol RDR (GM (CI))	0.040 (0.023–0.065)	0.041 (0.022–0.075)	0.907
<i>Streptococcus</i> spp.-% (GM (CI))	4.32 (2.79–6.69)	5.26 (3.73–7.43)	0.465
<i>Mycoplasma</i> spp.-% (GM (CI))	0.30 (0.14–0.64)	0.18 (0.09–0.39)	0.364
<i>Haemophilus</i> spp.-% (GM (CI))	4.83 (3.04–7.68)	4.87 (3.55–6.69)	0.974
FeNO ppb (GM (CI))	9.27 (6.37–13.49)	6.96 (4.95–9.83)	0.253
Sputum IL-8 pg/ml (GM (CI))	370 (249–549)	728 (578–916)	0.003
Sputum neutrophilic elastase ng/ml	13.1 $\pm$ 9.8	29.7 $\pm$ 21.5	0.003

Cut-off value for neutrophilia 55%.

Table 6. Results of the regression models, expressed as B values.

Dependent variable	Age	Pack years	FeNO	ACQ6
Sputum neutrophil-% (N = 50), B	1.60	–1.95	–0.30	9.29
	p = 0.038	p = 0.048	p = 0.070	p = 0.010
Sputum neutrophils per gram (N = 52), B	NS	NS	NS	NS
IL-8 (N = 52), B	0.02	NS	NS	NS
	p = 0.070			
Sputum neutrophils >55% (logistic) (N = 50), B	0.23	–0.23	NS	1.57
	p = 0.038	p = 0.081		p = 0.023

level of neutrophilic airway inflammation or the corresponding biomarker IL-8. Furthermore, no association between neutrophilic inflammation and the amount of common pathogenic bacteria was demonstrated in the airways. These findings are new, as no one to our knowledge has previously examined airway inflammation in steroid-free young smokers with asthma. The data of this study also indicated an association between age and neutrophils, even in these young patients, confirming results of other studies of older patient groups [11]. In addition, asthma symptom score was found to be positively associated with airway neutrophils, which may in smokers indicate a pathogenetic role for the neutrophils in asthma severity as suggested in earlier studies [24].

Former studies of asthma patients who smoke have shown heightened neutrophilic inflammation [8,9,25–27]. The smokers who have smoked for many years with a substantial number of pack years tend to have a higher daily consumption, which might be important for their degree of lung function impairment, reduced level of steroid sensitivity and possibly also airway pathology. However, the results of this study of young asthma patients indicate that the cumulated amount of tobacco smoked in early adulthood

may not leave a permanent mark on asthmatic neutrophilic airway inflammation. Thus, the increased airway neutrophilia more frequently present in asthmatic smokers than in non-smokers with asthma is perhaps, in the earlier years of life, primarily dependent on the presence of current tobacco smoke exposure. Hence, it could be speculated that the smoking-induced neutrophilia occurs through a switching-on mechanism when a threshold of tobacco exposure to the airways is reached. Whether such a process would be reversible after smoking cessation has not been fully elucidated, but is of considerable clinical interest. Some degree of reversibility in neutrophilia after smoking cessation has been demonstrated previously in a small study [28], whereas in a different study, neutrophils were higher in ex-smokers than in never-smokers [29].

Another important finding in this study was the observation that *Mycoplasma* spp., *Haemophilus* spp. and *Streptococcus* spp. were not significant predictors of airway neutrophilia, indicating a passive bacterial colonization process without inducing a chronic, local and immunological response. This is a novel finding in a population of young asthmatic smokers and somewhat surprising considering the pathological immunological mechanisms of bacterial

infections as well as the mitigating effect on neutrophilia induced with macrolide treatment [14]. However, the results of this study correspond well with those of an earlier study of non-smoking asthma patients [13] although, importantly, we did not find *Chlamydia* spp. in the airways of the participants in this study. Hence, the data indicate that airway neutrophilia in young smokers cannot be explained by colonization by known common airway bacterial pathogens.

The association between neutrophilia and IL-8 was found to be close in the present group of young adult smokers with asthma. On the other hand, an association between IL-8 and factors of possible importance for development of chronic reduced lung function was not found (correlation between IL-8 and FEV1% of expected: $R^2 = 0.002$, $p = 0.771$, not shown), in support of the univariate and logistic regression findings, in which sputum neutrophils was used as dichotomous variable, showing no difference in chronic airway impairment.

There are limitations to the interpretation of the results of this study. Fifty-two asthmatic subjects may not be a sufficient number to detect these correlations. However, when taking both the correlations and the regression analysis into account, none of the included factors, except for age and symptom score, appeared to systematically draw in any direction. Furthermore, this study included mainly asthmatics with relatively high baseline lung function. In a selected group of more severe asthma patients, different results may have been obtained. Moreover, acute effects of tobacco smoke could have influenced the results, in that time from last smoked cigarette may have differed between subjects.

It is also worth mentioning that in this study, the total load of bacteria was not only measured but also the proportion of signal for each species relative to the total signal load of each patient. Since no previous studies have examined the proportion of *Mycoplasma* spp., *Haemophilus* spp. and *Streptococcus* spp in asthmatics, further studies are needed to determine whether our findings can be generalized to other populations that might have a higher proportion of these bacteria. It could be speculated that not only the proportion of pathogenic species but also the absolute number of bacteria in the airways has an influence on airway neutrophilia. In addition, potentially, bacteria from the skin and mouth could have had an influence on the results. In regard to mouth pollution, this could indeed have been the case in that *Streptococcus* spp. were commonly found and were not further classified into subtypes. However, this does not seem to be a significant bias factor, when taking the low degree of squamous cells in the sputum samples is taken into account. Pollution from the skin, primarily lips, does not appear to have biased the results either, since no significant proportion of *Staphylococcus* spp., a frequent skin bacteria, was found.

Furthermore, airway neutrophilia in asthma is affected by other factors than those analyzed in this study. Infections in the lower respiratory tract lead to enhanced inflammation including eosinophils and neutrophils. Subjects with present or recent signs of airway infection were not included in the study, limiting this type of bias. However, the visits of some of the patients were performed in the wintertime, when common colds and other viral infections are prevalent.

Ongoing subclinical courses of infections may have increased the degree of airway neutrophilia.

Air pollution [30] and occupational exposures [31] to particulate matters have also been associated with airway neutrophilia. The concentration of these particles is highest in urban areas, in which the majority of the participants were resident and working. However, it seems that a quite high degree of air pollution is required to induce airway neutrophilia, at least when diesel particles, the main component of particulate matters, are considered [32].

In addition, obesity [33] has been shown to predispose for neutrophilic asthma. However, no statistical evidence for a body mass index effect on neutrophils was present in the data. Generally, the patients of this study were not overweight, and only five patients could be classified as obese (9.6%).

Furthermore, in the univariate analysis of this study, male gender was found to be insignificantly associated with airway neutrophilia, but this was not confirmed in the regression analysis. Thus, gender should not have had a biasing effect on the results.

Finally, previous studies have shown that airway neutrophilia in asthma is also associated with systemic inflammation with elevated levels of IL-6 and C-reactive protein [34,35], which was not found to be associated with smoking. However, it is not known whether systemic inflammation is a result of a spill-over effect from the respiratory system, or if it originates from other organs, causing increased inflammation in the airways.

To sum up, in this study, 52 young asthmatic smokers were examined for factors predicting airway neutrophilia. Using univariate and regression model statistics, tobacco history or presence of airway bacteria were found not to be significant predictors, indicating current smoking and the presence of asthma as found in other studies [8,9,20] to be the most important causes of airway neutrophilia in the young smokers with asthma. Future studies of larger study samples and including more factor variables are required to elucidate this further. Reversibility of the damaging neutrophilic airway inflammation remains a subject of interest.

Declaration of interest

The authors report no conflicts of interest.

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Notice of Correction

The version of this article published online ahead of print on 29 Jan 2014 contained an error in the Declaration of Interest. The sentence “The Respiratory Research unit has been provided an unrestricted grant from Pfizer Inc. and the Danish Lung Association in relation to the production of this article” should have read “The Respiratory Research unit has been provided an unrestricted grant from Pfizer Inc. for this study and the Danish Lung Association in relation to the study and to the production of the present paper.” The error has been corrected for this version.