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Changes in airway hyperresponsiveness following smoking cessation: Comparisons between Mch and AMP

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Summary
Background: Given the observed association between smoking, inflammation and airway hyperresponsiveness (AHR) one may predict that smoking cessation may improve AHR. However, only a few studies have investigated the effect of smoking on AHR and their results appear to be conflicting depending on the stimulus used in their bronchoprovocation protocol. The aim of the current study was to compare changes in AHR between direct (methacholine (Mch)) and indirect (adenosine 5′monophosphate (AMP)) stimuli before and at different time points after smoking cessation in smokers with allergic rhinitis (±asthma).
Methods: We have prospectively studied changes in AHR to inhaled Mch and AMP in smokers with allergic rhinitis (±asthma), before and at 6 and 12 months after smoking cessation.
Results: It was found that 28% (16/57) of the participants had quit smoking by the end of the study. No significant change in AHR was observed in smoking cessation failures. A significant improvement in AHR to AMP but not Mch was observed 6 months after smoking cessation in quitters; a 1.2 doubling concentrations change in PC_{20} AMP was measured whereas only a 0.4 doubling concentrations change was observed for PC_{20} Mch. However, after 12 months smoking cessation the improvement in AHR became significant for both AMP and Mch, their dose–response curves being displaced to the right to a similar extent (1.4 and 1.1 doubling concentrations for AMP and Mch, respectively).

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Conclusion: Smoking cessation can improve AHR in smokers who quit with a 6 months improvement being reported for the airways response to AMP but not Mch. AMP challenge may detect earlier changes in AHR in smokers during smoking cessation.

Introduction

Non-specific airway hyperresponsiveness (AHR), the abnormal increase in airflow limitation following exposure to non-allergic stimuli, is known to be associated with several chronic inflammatory conditions of the airways, including asthma and rhinitis.[1,2] AHR can be used to aid in diagnosis and characterization of individuals with inflammatory airway disease and appears to be a distinctive indicator of disease severity and disease progression.[3–5] Occurrence of non-specific AHR in individuals with asthma or rhinitis is likely to be a sign of ongoing inflammation in the lower airways.[6–8] Cigarette smoke induces inflammatory changes in the respiratory tract of individuals who smoke and particularly in those with diseased airways[9]; as a result cigarette smoke may further contribute to non-specific AHR. Given the observed association between smoking, inflammation and AHR, one may predict that smoking cessation (SC) may ameliorate AHR. Nevertheless, improvement in AHR may be strictly dependent on the stimulus used in bronchoprovocation protocols. AHR to indirect stimuli, such as inhaled adenosine 5′-monophosphate (AMP), appear to better reflect changes in airway inflammation than do AHR to direct bronchial provocants, such as methacholine (Mch).[10–12] This may lead to the speculation that bronchial provocation with inhaled AMP could provide a better tool for detecting inflammatory changes also in the context of a SC trial. In a cross sectional comparative study of active smokers and ex-smokers with COPD, active smokers exhibited a similar sensitivity to inhaled AMP and Mch whereas in ex-smokers much lower airway sensitivity to AMP was observed.[13] Differences in the level of AHR to AMP and Mch have also been detected when asthmatic smokers were compared to asthmatic nonsmokers.[14] These cross-sectional studies appear to suggest that SC ameliorates AHR to a different extent depending on the specific bronchostimulant used. However, detailed longitudinal studies are not available to confirm these cross-sectional findings. The aim of the current study was to compare changes in non-specific AHR to inhaled AMP and Mch before and at different time points after SC. As AHR is known to be frequently reported in subjects with allergic rhinitis and asthma, changes in AHR to inhaled AMP and Mch after quitting were investigated in smokers with seasonal allergic rhinitis with or without asthma. In addition, we have prospectively evaluated changes in lung function.

Methods and materials

A total of 57 active smokers (>15 cigarettes/day, for at least 5 yrs) with seasonal allergic rhinitis with or without asthma (age range 22–61), willing to quit, and with documented airways sensitivity to AMP ($PC20 < 800$ mg/ml) and Mch ($PC20 < 16$ mg/ml) were entered in the study. All were atopic, as defined by at least one positive skin prick test reaction (>3 mm wheal response) to a panel of 10 common allergens (including *Parietaria judaic*a, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Olea europea*, grass pollen, orchard, cypressus, alternaria, perennial rye, and cat allergen). Inclusion criteria comprised stable rhinitis/asthma (having had no exacerbation or respiratory tract infection in the previous 6 weeks) and baseline $FEV_1 \geqslant 70\%$ predicted. Subjects had never used systemic or topical corticosteroids before or had stopped their use at least 2 months before entry into the study. Throughout the study, only short acting inhaled β_2-adrenoreceptor agonists and oral antihistamines were allowed for relief of asthma/rhinitis symptoms (bronchodilators were withheld for 8 h before each bronchial challenge visits; antihistamines were not taken at least 3 days before each bronchial challenge visits). The study protocol was approved by the local hospital's ethics committee, and written informed consent was obtained for each patient.

This prospective study was designed as a 12-month longitudinal, observational, clinical trial to investigate the effect of quitting smoking on airway responsiveness to inhaled AMP and Mch in smokers with seasonal allergic rhinitis with or without asthma with documented AHR to both agonists.

Measurements of the AHR to inhaled AMP and Mch were carried out at baseline, at 6 and at 12 months after SC both in those smokers who will be still abstinent at time of sampling (quitters) and those who will fail to quit smoking (SC failures). SC failures were used in this study as a smoking reference group for comparison between groups after smoking cessation. The study comprised the following visits (Figure 1).

Baseline/screening visit

Subjects undertook concentration–response studies with inhaled Mch first followed by AMP challenge 2–3 h later. The order of inhalation challenges was kept identical for all subjects throughout the 12 months of study. This visit took place outside the pollen season. Lung function (FEV_1, flow volume curve (FVC)) was assessed by spirometry. Evaluation of small airways function was carried out by deriving from the FVC taken at baseline the forced expiratory flow at the middle half of the FVC ($FEF_{25-75\%}$). A detailed smoking history was taken and individual pack-years calculated (pack-years = [total number of years of cigarettes consumption] × [total number of cigarettes smoked per day]/20) together with scoring of their level of nicotine dependence by means of a standard FTND questionnaire.[15] Concentration of carbon monoxide in expired breath (eCO) was also measured (Micro CO-Micro Medical Ltd., Rochester,

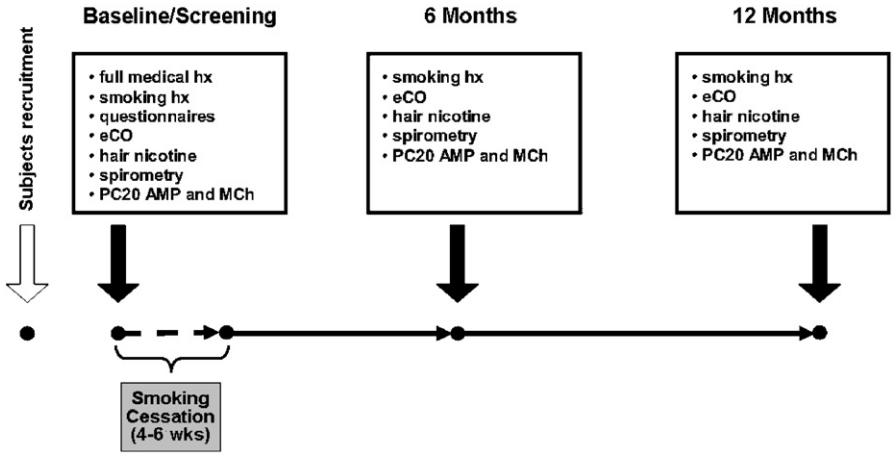

Figure 1 Schematic diagram of the study design. Smokers willing to quit took part in a longitudinal study to assess changes in AHR to direct (MCh) and indirect (AMP) stimuli at baseline and prospectively at 6 and 12 months after smoking cessation. Smoking cessation failures are used as smoking reference group for the change in AHR to MCh and AMP after smoking cessation. eCO: carbon monoxide in expired breath; MCh: methacholine; AMP: adenosine 5′-monophosphate.

UK) and a small hair sample taken for nicotine assessment (about 25 mg). Subjects were invited to take part in a SC program; they were instructed on how to prepare to stop smoking and to set a quit date, prescribed with medications tailored to their individual needs, and booked for their first follow-up visit within 7 days.

Follow-up visits

Between the *baseline* visit and the 6 month visit, SC counselling and treatment were offered over several brief appointments (5–8 appointments within the first 8 weeks of the SC programme). On each occasions, participants reported the quantity of medication used and their smoking status. eCO readings were taken at each appointment.

Six months visit

Six ($\pm$1; to allow for this visit to take place outside the pollen season) months after admission, an eCO reading was recorded and a further hair sample taken. Subjects undertook concentration–response studies with inhaled Mch first followed by AMP challenge 2–3 h later. As before, the order of inhalation challenges was kept identical. FEV_1, FVC and $FEF_{25-75\%}$ were noted.

Twelve months visit

Twelve ($\pm$1; to allow for this visit to take place outside the pollen season) months after admission, a final eCO reading and hair sample were taken. Similar to the 6 month visit, subjects undertook concentration–response studies with inhaled Mch first followed by AMP challenge 2–3 h later and spirometry parameters (FEV_1, FVC, and $FEF_{25-75\%}$) were recorded.

The SC strategies/interventions currently in use at the University of Catania for smokers taking part in the study follow the clinical practice guidelines on SC of the AHCPR[16] and was specifically modified to facilitate subjects' participation and to improve SC specialists' performance. Typically, our SC program has the duration of 4–6 weeks. Participants are instructed on how to prepare to stop smoking and encouraged to set a "quit date" right away. A variety of methods are employed including motivational counselling, nicotine replacement therapy (NRT) and the prescription of medications, with each intervention being tailored to individual needs. Treatment management is designed to match patients' level of nicotine dependence and dose, to control for anxiety and depression, and takes into account the level of motivation. In general, pharmacological treatment includes the use of assorted NRT in association with bupropion (300 mg/day). The first follow-up visit is timed within a few days from the established "quit date". Further SC counselling by motivational interviewing is offered at each following visits (usually up to 6–8 weeks). Abstinence from cigarette smoking is objectively assessed by measuring the concentration of eCO at each appointment and by assaying the level of hair nicotine at 6 and 12 months after admission in the SC programme. Participants who had given up smoking with a eCO concentration of <5 ppm at each follow up appointment and/or low levels (<5 ng/mg hair—this cutoff threshold was derived from the data of a random group of 50 healthy long life non smokers considering their median [inter-quartile range] nicotine levels was 2.60 [1.51; 3.95] ng/mg hair) of hair nicotine at 6 and 12 months after SC were defined as quitters. Those smokers who failed to meet these criteria were categorized as SC failures.

Samples were collected by cutting hair from the base of the scalp behind the ear and were stored in paper envelopes. About 1 cm of hair was then cut from the end closest to the scalp using a scalpel swabbed with methanol. Hair was handled with tweezers and fresh paper was used underneath each sample. Duplicate samples of about 2–4 mg were weighed into capped 10-ml polypropylene tubes and then washed for 90 min in 2 ml of dichloromethane at room temperature. The dichloromethane was aspirated off using a polypropylene transfer pipette and samples dried briefly at 50 °C. The hair was then digested at 50 °C overnight in 2 ml

of 1 M NaOH in capped tubes. The digests were allowed to cool and 50 μl of 750 μg/l 2-phenylimidazole was added as an internal standard. Nicotine was then extracted into 4 ml of diethyl ether in the capped tube by vortexing for 40–60 s. After transferring the ether to a second 10-ml polypropylene tube containing 100 μl of 0.1% (v/v) HCl in methanol, the ether was evaporated to dryness under a stream of air at 40 °C. The extract was then injected into the gas cromathography instrument (Trace GC Ultra by Thermo Electron Corporation) with a nitrogen phosphorus detector (NPD). The sample was volatilized at the injection port and eluted through a non-polar capillary column (SPB-5 by Supelco) under increasing temperature. As the sample moved through the column, various components were separated due to their affinity for the stationary phase of the column and could be identified by their distinct retention time. Samples for use as quality controls were acquired from non-smoking volunteers when they had routine haircuts. The samples were cut into 0.5–1.0 cm lengths and then randomised by mixing. These samples were run randomly throughout batches. A standard extracted without hair was also run in each batch to specifically check the extraction and chromatographic part of the procedure. With this method the estimated lower limit of detection is 0.2 ng nicotine/mg hair and the mean intra-assay variation is approximately 7.5%. All assays were performed without knowledge of smoking habits.

Spirometry was carried out according to the European Society Guidelines using the European Community for Steal and Coal reference equations.[17] AHR was evaluated by Mch and AMP bronchial challenge, as described previously.[18] In brief, Mch (Lofarma, Milan, Italy) and AMP (Sigma Chemical Co., St. Louis, MO, USA) were dissolved in PBS (pH 7.4) and normal saline respectively to produce increasing doubling concentrations (0.06–16 mg/ml for Mch; 3125–800 mg/ml for AMP). Solutions were administered as aerosols generated from a starting volume of 3 ml in a disposable Inspiron Minineb (C.R. Bard International, Sunderland, UK) driven by compressed air at 8 l/min. Patients inhaled increasing doubling concentrations of agonist in five breaths from functional residual capacity to total lung capacity via a mouthpiece and FEV_1 measured at 1 and 3 min after each administration. The challenges were stopped when a decrease of 20% in FEV_1 had been achieved or when the maximum concentration of agonist had been inhaled. The bronchial responses to the inhaled agonists were expressed as the provocative concentration causing a 20% decline in FEV_1 (PC_{20}), which was calculated by linear interpolation from the concentration–response curve constructed on a logarithmic scale.

Sample size calculation for this study was based from previous clinical pharmacological studies with Mch and AMP bronchial challenges.[10,16] Hence, sample size calculations indicate that a total of 15 subjects will be required to detect at least 1 doubling concentration difference between two time periods with a power of 80% and using a significance level of 5%. Allowing for possible drop out and considering the SC rate of 30% in our clinic, the sample size was then increased to a target of 55–60 subjects. As a 20% fall in FEV_1 could not be obtained in a number of participants at the highest concentration of agonists administered, a conservative estimate was obtained by assigning a value of twice the highest concentration given. Because of these censored data, this set of PC_{20} data could not be considered normal and were compared for significance using non-parametric statistical analysis.

For PC_{20} data available from participants who completed all visits after SC, Friedman tests were used to compare median PC_{20} values across all three time periods at once (baseline, 6 and 12 months) with each agonist (AMP and Mch) analysed separately. Non-parametric post hoc tests (i.e. Wilcoxon's Signed Ranks test) were then used to compare median PC_{20} between pairs of time periods (baseline vs. 6 months, 6 vs. 12 months) if a significant difference in medians between all three time periods was observed. As the distribution of FEV_1, FVC and FEF25–75 could be considered to be normally distributed, repeated measures ANOVA was used to compare mean FEV_1, FVC and FEF25–75 values across all three time periods (baseline 6 and 12 months); each group of patients (quitters and relapsers) was analysed separately. Paired *t*-tests were used to compare mean values between pairs of time periods (baseline vs. 6 months, 6 vs. 12 months) if a significant difference in means between all three-time periods was observed. The PC_{20} data available from all smokers at baseline (every subject had an uncensored value for both AMP and Mch PC_{20} in this data set) were logarithmically transformed to improve normality and linear regression was performed using the following predictors: age, gender and pack/years for each agonist separately. Each predictor was assessed individually for its ability to predict pack/years, and then multiple linear regression was performed as appropriate. All analyses were performed with the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL) for Windows version 12.0. A two-tailed *p*-value of less than 0.05 was considered to indicate statistical significance.

Results

Throughout the SC program 27 subjects failed to return for their follow-up visits. Thus, complete data were available from 30 smokers with seasonal allergic rhinitis (±asthma) after 1-year SC program, of which 16 quitters and 14 SC failures. Asthma was also seen in association with allergic rhinitis in three quitters and three relapsers. Data from subjects who did not complete the study or were lost to follow-up were not being included in the analyses, with the exception of the baseline PC_{20} data used to perform linear regression analyses. Descriptive characteristics of this study population are outlined in Table 1.

Abstinence from cigarette smoking was objectively confirmed at 6 and 12 months after admission in the SC programme by biochemical validation of hair nicotine (<5 ng/mg hair) and by eCO monitoring (<5 ppm). For the sustained quitters, comparisons with baseline eCO and hair nicotine values showed a significant and substantial reduction at 6 ($P<0.001$), and 12 months ($P<0.001$) after SC, whereas no significant change were observed at any time point in the SC failures. For those who successfully quitted, the geometric mean eCO readings at baseline decreased from 24.2 to 3.8 ppm at 6 months and to 3.1 ppm at 12 months. Likewise, the median hair nicotine value at baseline

Table 1 Subjects' characteristics at baseline.

	Quitters	Cessation failures	Lost to follow up
No. of subjects	16	14	27
Sex (male:female ratio)	8:8	8:6	10:17
Age (yrs)	39.4 (±4.4)	40.7 (±2.9)	38.5 (±4.0)
Duration of rhinitis (yrs)	9.7 (6–19)	11.0 (7–24)	10.3 (5–25)
No. of subjects with a diagnosis of asthma	3	3	–
FEV_1 (% predicted)	90.5 (±5,2)	92.0 (±6,1)	89.2 (±5,5)
FEV_1/FVC	75.7 (±2.6)	78.1 (±2,4)	76.2 (±3,3)
$FEF_{25-75\%}$ (% predicted)	72.6 (±6,9)	75.0 (±6,1)	70.1 (±7,9)
Geom. Mean (range) PC_{20} MCh (mg/ml)	2.88 (0.10–13.30)	4.08 (0.71–14.90)	3.54 (0.50–13.80)
Geom. Mean (range) PC_{20} AMP (mg/ml)	90.9 (9.6–377.6)	106.8 (9.7–319.7)	160.4 (21.3–445.2)
Pack/years	43.9 (±9.0)	39.7 (±11.1)	40.9 (±12.5)
Exhaled CO (ppm)	24.2 (±4.1)	27.5 (±5.0)	23.0 (±4.7)
Hair nicotine (ng/mg hair)	72.0 (42.2; 115.9)	79.5 (47.3; 134.5)	73.5 (41.0; 123.3)
FTND	6.3 (±0.5)	6.7 (±0.4)	6.8 (±0.6)

AMP: adenosine 5′-monophosphate.
Mch: methacholine.
CO: carbon monoxide.
FTND: Fagerstrom test for nicotine dependence.
Age, FEV_1, FEV_1/FVC, $FEF_{25-75\%}$, pack/years, exhaled CO, and FTND are expressed as mean (±SEM) duration of rhinitis is expressed as mean (range).
PC_{20} methacholine and PC_{20} AMP values are expressed as geometric mean (range).
Hair nicotine is expressed as median (inter-quartile range).

decreased from 72.0 to 3.3 ng/mg hair at 6 months, and to 2.1 ng/mg hair at 12 months.

Intent-to-treat analyses, in which subjects not available for the 12-month follow-up were considered SC failures, found that 28% (16/57) of the participants had quit smoking by the end of the study. It is likely that interventions in the context of a research setting may well contribute to the high rate of sustained cessation observed in this study.

For the quitters, comparisons with baseline PC_{20} AMP values showed a significant difference both at 6 ($P = 0.006$), and 12 months ($P = 0.002$) after SC, whereas no significant change in PC_{20} AMP values was observed at any time point in the SC failures. For those who successfully quitted, the geometric mean PC_{20} AMP value at baseline increased from 90.9 mg/ml (range, 9.6–377.6 mg/ml) to 203.8 mg/ml (range, 28.0–800.0 mg/ml) at 6 months, and to 239.1 mg/ml (range, 30.0–800.0 mg/ml) at 12 months (Table 2). When changes in PC_{20} AMP values over time were expressed as doubling dilutions, mean±SEM changes of 1.2±0.3, and 1.4±0.3 doubling concentrations were shown at 6 and 12 months, respectively (Figure 2A). For those who relapsed during the SC program, the geometric mean PC_{20} AMP values were 106.8 mg/ml (range, 9.7–319.7 mg/ml) at baseline, 120.5 mg/ml (range, 7.9–800.0 mg/ml) at 6 months, and 129.9 mg/ml (range, 9.5–800.0 mg/ml) at 12 months (Table 2). Similar findings were obtained when asthmatics ($n = 3$) were excluded from analyses (data not shown).

For the quitters, comparisons with baseline PC_{20} Mch values showed a significant difference only at 12 months ($P = 0.002$) after SC; no significant change in PC_{20} Mch values was observed at any time point in the SC failures. For those who successfully quitted, the geometric mean PC_{20} Mch value at baseline increased from 2.88 mg/ml (range, 0.10–13.30 mg/ml) to 3.87 mg/ml (range, 0.16–16.00 mg/ml) at 6 months, and to 6.04 mg/ml (range, 0.24–16.00 mg/ml) at 12 months (Table 3). When changes in PC_{20} Mch values over time were expressed as doubling dilutions, mean±SEM changes of 0.4±0.2, and 1.1±0.2 doubling doses were shown at 6 and 12 months, respectively (Figure 2B). For those who relapsed during the SC program, the geometric mean PC_{20} Mch values were 4.08 mg/ml (range, 0.71–14.90 mg/ml) at baseline, 5.03 mg/ml (range, 1.02–16.00 mg/ml) at 6 months, and 5.37 mg/ml (range, 1.32–16.00 mg/ml) at 12 months (Table 3). Equivalent results were obtained when asthmatics ($n = 3$) were excluded from analyses (data not shown).

For PC_{20} AMP, the model was found to be significantly different from the null model (a model containing only a constant term). About 70% of the variations in the data were explained by the model containing age, gender and pack-years. Pack/years was found to be significantly associated with the level of AHR to AMP ($P < 0.001$) (Figure 3A). Subjects with high numbers of pack-years were found to have lower values of PC_{20} AMP. Age and gender were not found to be significant predictors of PC_{20} AMP after controlling for the effect of pack-years. Residual analysis showed two residuals had a z-value greater than 2 or less than −2 (4%) which is acceptable. Residuals were also found to be approximately normally distributed which is one of the assumptions that regression is based on.

For PC_{20} Mch, the model was found to be just marginally significantly different from the null model ($P = 0.042$) with only 11% of the variation in the data was explained by the model containing age, gender and pack-years. Pack/years was found to be a marginally significant predictor ($P = 0.033$) of PC_{20} Mch (Figure 3B). Age and gender were not found to be significant predictors of PC_{20} Mch after controlling for the effect of pack/years. Residual analysis

Table 2 Individual PC_{20} AMP values before and after smoking cessation in quitters and cessation failures.

Quitters	PC_{20} AMP (mg/ml)			Cessation failures	PC_{20} AMP (mg/ml)		
	Baseline	6 Months	12 Months		Baseline	6 Months	12 Months
1	299.0	476.4	570.5	1	319.7	513.0	800.00
2	242.2	800.0	800.0	2	150.9	211.8	254.61
3	345.0	800.0	625.3	3	213.4	150.3	130.63
4	377.6	352.9	400.8	4	202.8	157.2	181.46
5	50.5	63.4	76.6	5	230.1	172.5	207.70
6	164.2	318.3	388.2	6	82.8	103.4	124.08
7	143.3	590.5	408.4	7	51.0	81.1	57.42
8	243.5	800.0	800.0	8	298.6	800.0	563.67
9	210.6	393.0	800.0	9	115.7	140.0	168.98
10	306.0	120.1	174.4	10	301.9	160.6	182.72
11	14.4	159.7	121.7	11	31.3	73.4	88.11
12	21.5	50.3	160.4	**12**	**9.7**	**7.9**	**9.50**
13	160.0	643.8	572.6	**13**	**95.7**	**87.6**	**55.37**
14	**16.9**	**28.0**	**30.0**	**14**	**40.6**	**39.9**	**67.90**
15	**15.5**	**40.6**	**58.6**				
16	**9.6**	**31.2**	**37.5**				
G.Mean (Range)	90.9 (9.6–377.6)	203.8 (28.0–800.0)	239.1 (30.0–800.0)		106.8 (9.7–319.7)	120.5 (7.9–800.0)	129.9 (9.5–800.0)

AMP: adenosine 5'-monophosphate.
PC_{20} AMP values are expressed as geometric mean (range).
PC_{20} values in bold are for rhinitic subjects with asthma.

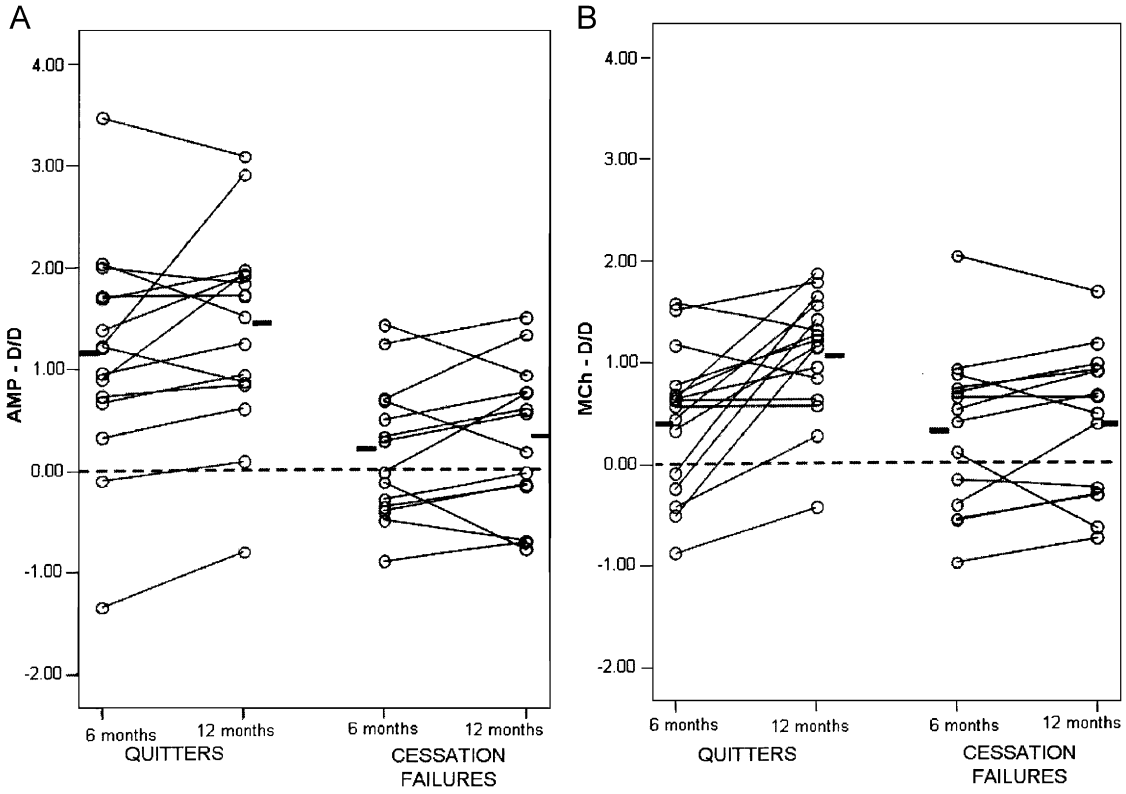

Figure 2 Individual changes in baseline PC_{20} AMP (A) and PC_{20} Mch (B) values 6 and 12 months after smoking cessation in quitters ($n = 16$) and smoking cessation failures ($n = 14$). Changes in PC_{20} values from baseline (i.e. dotted line) are expressed as mean $\pm$ SEM doubling dilutions (D/D). Means are shown as horizontal bars. For AMP a significant difference ($P < 0.01$) was observed both at 6 and 12 months after smoking cessation (quitters vs relapsers), whereas for Mch a significant change ($P < 0.01$) was observed only at 12 months.

Table 3 Individual PC_{20} Mch values before and after smoking cessation in quitters and cessation failures.

Quitters	PC_{20} MCh (mg/ml)			Cessation failures	PC_{20} MCh (mg/ml)		
	Baseline	6 Months	12 Months		Baseline	6 Months	12 Months
1	12.10	6.58	8.95	1	10.50	7.08	8.45
2	2.15	3.67	4.97	2	3.21	6.07	7.20
3	10.86	16.00	16.00	3	4.31	7.12	8.04
4	13.30	9.96	16.00	4	8.80	16.00	12.22
5	2.67	2.26	7.12	5	12.30	9.22	16.00
6	3.85	8.64	6.84	6	2.30	9.44	7.39
7	2.25	3.05	6.63	7	2.80	4.49	5.48
8	10.40	16.00	16.00	8	14.90	16.00	9.53
9	1.04	2.98	3.58	9	2.30	2.05	1.94
10	5.37	8.36	10.30	10	4.02	5.30	6.36
11	5.10	4.80	16.00	11	7.55	3.80	4.50
12	4.05	12.16	10.06	**12**	**0.71**	**1.02**	**1.32**
13	4.50	5.66	9.96	**13**	**4.72**	**3.21**	**3.81**
14	**0.10**	**0.16**	**0.24**	**14**	**1.02**	**1.59**	**1.59**
15	**0.66**	**1.03**	**2.42**				
16	**0.71**	**0.50**	**1.56**				
G.Mean (Range)	2.88 (0.10–13.30)	3.87 (0.16–16.00)	6.04 (0.24–16.00)		4.08 (0.71–14.90)	5.03 (1.02–16.00)	5.37 (1.32–16.00)

Mch: methacholine.
PC_{20} MCh values are expressed as geometric mean (range).
PC_{20} values in bold are for rhinitic subjects with asthma.

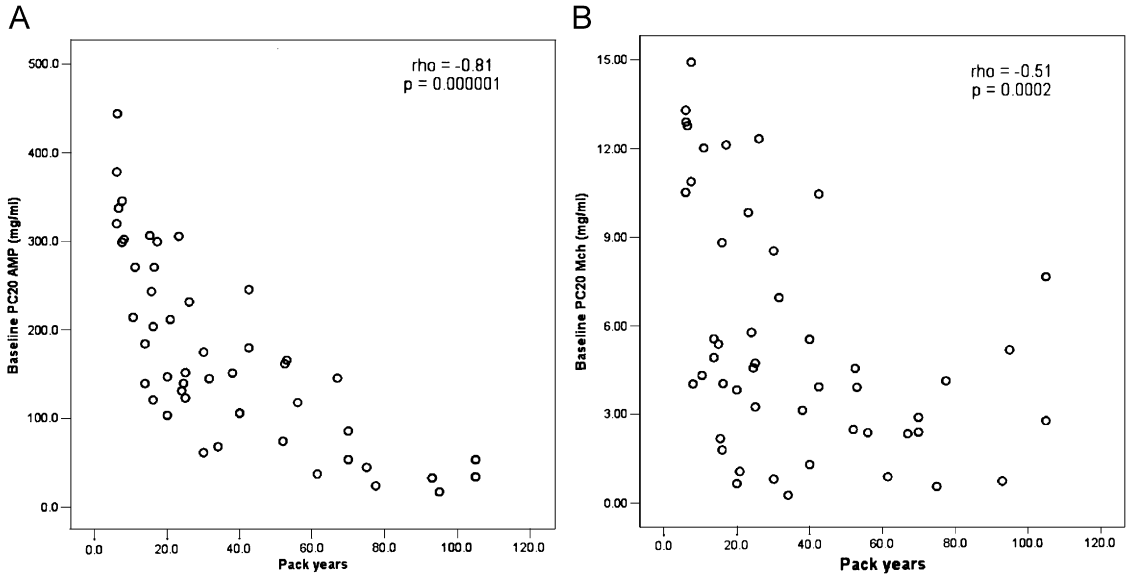

Figure 3 Univariate correlation of PC_{20} AMP (A) and PC_{20} Mch (B) values at baseline with number of pack/years in smokers with allergic rhinitis ($n = 57$). The strength of the association was assessed using Spearman's rho. Although pack/years were found to be significantly associated with the level of AHR to both AMP and Mch, the strength of the association was much stronger for AMP.

showed 2 residuals had a *z*-value greater than 2 or less than −2 (4%) which is acceptable. Residuals were also found to deviate slightly from a normal distribution. No significant correlations were found between changes in PC_{20} AMP or in PC_{20} Mch after SC and pack/years.

For the quitters, repeated measures ANOVA for $FEF_{25-75\%}$ values indicated that there was a highly significant difference between all 3 time periods ($P<0.001$). Paired *t*-tests did not show a significant difference between baseline and 6 months ($P = 0.17$) but did show a highly significant difference between 6 and 12 months ($P<0.001$); repeated measures ANOVA did not show a significant difference between all three time periods ($P = 0.13$) in the SC failures. Repeated measures ANOVA for FEV_1 and FVC

failed to show any significant difference between all three-time periods in the study groups. Statistical analysis failed to reveal any significant association between changes in PC_{20} AMP or in PC_{20} Mch after SC and spirometry data.

Discussion

In the present study, we have shown improvement in AHR in active smokers with seasonal allergic rhinitis with or without asthma after SC. Our SC program resulted in a high rate (approximately 30%) of sustained cessation among smokers due to the intensity and duration of the program, along with the extended use of pharmacological treatment together with motivational interviewing. Though by the end of the study changes in AHR to AMP and Mch improved to the same extent, improvement in AHR 6 months after SC was observed only for AMP and not Mch.

Intuitively one would expect that the intensity of smoking is associated with the severity of AHR in smokers. Indeed, we have also shown that the overall intensity of smoking exposure (assessed as pack/years) is related to the severity of AHR. In particular, the analysis of the cross-sectional data has revealed that pack/years is more strongly associated with AHR to AMP than Mch.

Early studies on the effect of SC on AHR to direct stimuli in 'healthy' smokers have produced inconsistent findings.[19–22] This may be depending on a number of reasons including the small number of subjects investigated, the lack of suitable controls, and the nature of the provoking stimulus used in bronchoprovocation protocols. Indeed, our longitudinal study has shown substantial improvement in AMP and Mch airways responsiveness 1-year after SC in smokers for whom an objective proof of cessation was obtained. Furthermore, one of the strength of the present study is that the beneficial effects of SC in our quitters were compared with individuals who relapsed thus strengthening our conclusions that the observed improvement in AHR is a consistent finding.

The observed improvement in AHR after SC has important clinical implications. For instance, independently of their smoking status, individuals with documented Mch reactivity are known to be at risk for new onset asthma, cough symptoms and decreased levels of pulmonary function.[23–26] Moreover, we observed an increased risk (OR, 5.9) of new onset asthma among current smokers compared to never smokers and, in the multivariate model adjusting for other asthma risk factors, this risk appeared to increase with each pack/years unit.[27] Thus, improving AHR in smokers can avoid unnecessary morbidity.

Cross-sectional comparative studies in patients with COPD have shown that smokers have similar levels of AHR to Mch as ex-smokers,[13,28,29] whereas the level of airway sensitivity to inhaled AMP is reported to be more severe in smoking than in ex-smoking COPD patients.[13] This is in agreement with recent observations in subjects with allergic rhinitis who smoked; compared to nonsmokers, smokers exhibit a much greater sensitivity to AMP.[30] Unfortunately, the cross-sectional methodology does not allow elucidating the temporal nature of the relationship between cigarette smoking and changes in AHR. The strength of evidence is usually developed from prospective cohort studies and the present longitudinal study clearly illustrate that 6 months after SC AHR to AMP is reduced to a larger degree compared to Mch whereas changes in AHR improved to the same extent for both AMP and Mch 12 months after smoking cessation. These findings are to some extent compatible with those of a recent longitudinal study in COPD patients, showing an estimated two doubling concentrations improvement in AMP and Mch responsiveness 1-year after SC in quitters with COPD, with improvements in PC_{20} Mch being less consistent than PC_{20} AMP.[31]

The observation that SC at 6 months reduced sensitivity to AMP, whereas no significant effect on AHR to direct stimuli was observed, is novel. The explanation for the greater effect of SC on AMP over Mch is not known, but must relate to their different mechanism or mechanisms of action. Whereas Mch acts directly on airway smooth muscle cells via binding to muscarinic receptors, AMP appears to mediate bronchoconstriction indirectly via stimulation of specific adenosine A2B receptors on airway mast cells, with subsequent release of preformed and newly formed mediators.[32]

The observed improvement in AHR to AMP after SC might result from a decrease in the number or in the activation state of airway mast cells. Indeed, it has been demonstrated that the number of mast cells in the bronchial biopsies obtained from never smokers is much reduced compared to asymptomatic smokers.[33] Interestingly, the extensive mast cell infiltration exhibited by asymptomatic smokers at all levels of their bronchial mucosa was particularly predominant within the airway smooth muscle compartment.[34] Smoke exposure has been shown to induce activation/degranulation in airway resident mast cells[35,36] and elevated levels of histamine and tryptase have been measured in the bronchoalveolar lavage fluid of active smokers.[37] Besides mast cell priming in vivo, it has been hypothesized that the airways response to AMP may reflect the dynamic balance between the distribution and affinity of the high affinity A2A receptors and that of the low-affinity A2B receptors present on mast cells.[38] Indeed, cigarette smoking may upregulate the activity of A2B receptors expressed in mast cells and macrophages, as recently shown by Varani et al.[39] in their elegant work in COPD patients.

Cigarette smoke may also have a direct effect on airway smooth muscle responsiveness. Experiments performed in isolated human bronchi showed that constituents of cigarette smoking altered airway smooth muscle responsiveness in a dose-dependent manner.[40] In guinea pigs, smoke exposure significantly increased histamine induced bronchoconstriction but not acetylcholine induced bronchoconstriction, indicating some specificity of the mediators involved in the AHR triggered by smoke exposure.[41] In addition to affecting airway smooth muscle responsiveness directly, smoking exposure can prime smooth muscle cells to exogenous stimuli with enhanced AHR by releasing cytokines and proteases from airway mast cells. There is ample evidence that several mast cell mediators including tryptase and TNFalfa may produce further sensitization of the airway smooth muscle to a number of contractile stimuli.[42,43]

Whatever the mechanism accounting for the earlier effect of SC on AMP over Mch, it is apparent that responsiveness of the airways to inhaled AMP may be also used as a sensitive indicator in smoke-induced inflammatory

changes. This view is also supported by our cross-sectional observation that the overall level of smoking exposure (assessed as pack/years) is more strongly associated with AHR to AMP than Mch at baseline.

$FEF_{25-75\%}$ values at baseline indicate that a reduction in maximum mid-expiratory flow rate—a spirometric manifestation of small airways disease-is present in our study population. This is not surprising considering that the majority of participants were heavy smokers and all had allergic rhinitis.[44,45] Nonetheless, a novel observation from the current study is that an improvement in $FEF_{25-75\%}$ values was observed at 12 months in those active smokers who successfully completed the SC program whereas no change was detected in the SC failures. This pattern of response is similar to that described for PC_{20} Mch. However, statistical analysis failed to reveal any significant association between changes in PC_{20} Mch after SC and $FEF_{25-75\%}$ data. Larger clinical trials are required to shed more light on the relevance of these findings in relation to smoking cessation.

Our prospective study in smokers with allergic rhinitis ($\pm$asthma) now shows that SC can also improve AHR in these individuals and this may confer some potential clinical benefit.

Although this improvement was similar for both direct and indirect agonists at the end of the study, an improvement at 6 months after SC was observed only for AMP but not Mch. This suggests that monitoring of AHR to AMP might be an accurate guide to detect earlier changes in AHR in smokers during SC.

Despite its complexity, this research study should reinforce the public health imperative to decrease exposure to tobacco products. If more successful strategies for preventing tobacco use could be developed, the burden of smoking-related diseases in the population could be significantly reduced.

Conflict of interest statement

None of the authors have a conflict of interest to declare in relation to this work.

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References

1. Sterk PJ, Fabbri LM, Quanjer PH, et al. Airway responsiveness. Standardized challenge testing with pharmacological, physical and sensitizing stimuli in adults. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. *Eur Respir J Suppl*. 1993 March;16:53–83.
2. Townley RG, Ryo UY, Kolotkin BM, et al. Bronchial sensitivity to methacholine in current and former asthmatic and allergic rhinitis patients and control subjects. *J Allergy Clin Immunol* 1975;**56**(6):429–42.
3. Gibson PG, Saltos N, Borgas T. Airway mast cells and eosinophils correlate with clinical severity and airway hyperresponsiveness in corticosteroid-treated asthma. *J Allergy Clin Immunol* 2000; **105**(4):752–9.
4. Braman SS, Barrows AA, DeCotiis BA, et al. Airway hyperresponsiveness in allergic rhinitis: a risk factor for asthma. *Chest* 1987;**91**:671–4.
5. Polosa R, Li Gotti F, Mangano G, et al. Effect of immunotherapy on asthma progression, BHR and sputum eosinophils in allergic rhinitis. *Allergy* 2004;**59**(11):1224–8.
6. Djukanovic R, Lai CK, Wilson JW, et al. Bronchial mucosal manifestations of atopy: a comparison of markers of inflammation between atopic asthmatics, atopic nonasthmatics and healthy controls. *Eur Respir J* 1992;**5**(5):538–44.
7. Polosa R, Ciamarra I, Mangano G, et al. Bronchial hyperresponsiveness and airway inflammation markers in non-asthmatics with allergic rhinitis. *Eur Respir J* 2000;**15**:30–5.
8. Polosa R, Li Gotti F, Mangano G, et al. Monitoring of seasonal variability in bronchial hyper-responsiveness and sputum cell counts in non-asthmatic subjects with rhinitis and effect of specific immunotherapy. *Clin Exp Allergy* 2003;**33**(7): 873–81.
9. van der Vaart H, Postma DS, Timens W, et al. Acute effects of cigarette smoke on inflammation and oxidative stress: a review. *Thorax* 2004;**59**(8):713–21.
10. Prosperini G, Rajakulasingam K, Cacciola RR, et al. Changes in sputum counts and airway hyperresponsiveness after budesonide: monitoring anti-inflammatory response on the basis of surrogate markers of airway inflammation. *J Allergy Clin Immunol* 2002;**110**(6):855–61.
11. Joos GF, O'Connor B, Anderson SD, et al. ERS task force. Indirect airway challenges. *Eur Respir J* 2003;**21**(6):1050–68.
12. Russo C, Arcidiacono G, Polosa R. Adenosine receptors: promising targets for the development of novel therapeutics and diagnostics for asthma. *Fundam Clin Pharmacol* 2006;**20**(1): 9–19.
13. Oosterhoff Y, de Jong JW, Jansen MA, et al. Airway responsiveness to adenosine 5′-monophosphate in chronic obstructive pulmonary disease is determined by smoking. *Am Rev Respir Dis* 1993;**147**(3):553–8.
14. Oosterhoff Y, Jansen MA, Postma DS, et al. Airway responsiveness to adenosine 5′-monophosphate in smokers and nonsmokers with atopic asthma. *J Allergy Clin Immunol* 1993;**92**(5): 773–6.
15. Heatherton TF, Kozlowski LT, Frecker RC, et al. The Fagerstrom test for nicotine dependence: a revision of the Fagerstrom tolerance questionnaire. *Br J Addict* 1991;**86**:1119–27.
16. Fiore MC, Bailey WC, Cohen SJ, et al. Smoking cessation. Clinical Practice Guideline No 18. Rockville, Maryland: US Department of Health and Human Services, Public Health Service, Agency for Health Care Policy and Research, April 1996, AHCPR Publication No 96-0692.
17. Quanjer PhH, Tammeling GJ, Cotes JE, et al. Standardized lung function testing. *Eur Respir J* 1993;**6**:1–99.
18. Polosa R, Lau LC, Holgate ST. Inhibition of adenosine 5′-monophosphate- and methacholine-induced bronchoconstriction in asthma by inhaled frusemide. *Eur Respir J* 1990;**3**(6): 665–72.
19. Buczko GB, Day A, Vanderdoelen JL, et al. Effects of cigarette smoking and short-term smoking cessation on airway responsiveness to inhaled methacholine. *Am Rev Respir Dis* 1984; **129**:12–4.

20. Simonsson BG, Rolf C. Bronchial reactivity to methacholine in ten non-obstructive heavy smokers before and up to one year after cessation of smoking. *Eur J Respir Dis* 1982;**63**:526–34.
21. Israel RH, Ossip-Klein DJ, Poe RH, et al. Bronchial provocation tests before and after cessation of smoking. *Respiration* 1988;**54**:247–54.
22. Lim TK, Taylor RG, Watson A, et al. Changes in bronchial responsiveness to inhaled histamine over four years in middle aged male smokers and ex-smokers. *Thorax* 1988;**43**:599–604.
23. Sparrow D, O'Connor G, Colton T, et al. The relationship of nonspecific bronchial responsiveness to the occurrence of respiratory symptoms and decreased levels of pulmonary function: the Normative aging study. *Am Rev Respir Dis* 1987; **135**:1255–60.
24. Hopp RJ, Townley RG, Biven RE, et al. The presence of airway reactivity before the development of asthma. *Am Rev Respir Dis* 1990;**141**:2–8.
25. Tashkin DP, Altose MD, Connett JE, et al. Methacholine reactivity predicts changes in lung function over time in smokers with early chronic obstructive pulmonary disease. The Lung Health Study Research Group. *Am J Respir Crit Care Med* 1996;**153**(6 Part 1):1802–11.
26. Polosa R, Li Gotti F, Mangano G, et al. Effect of immunotherapy on asthma progression, BHR and sputum eosinophils in allergic rhinitis. *Allergy* 2004;**59**(11):1224–8.
27. Sarvà M, Al-delaimy WK, Russo C, et al. Greater risk of incident asthma cases in adults with allergic rhinitis: importance of cigarette smoking. *Eur Respir J* 2006;**28**(Suppl. 50):798s.
28. Postma DS, Renkema TE, Noordhoek JA, et al. Association between nonspecific bronchial hyperreactivity and superoxide anion production by polymorphonuclear leukocytes in chronic air-flow obstruction. *Am Rev Respir Dis* 1988;**137**:57–61.
29. Kanner RE, Connett JE, Altose MD, et al. Gender difference in airway hyperresponsiveness in smokers with mild COPD. The Lung Health Study. *Am J Respir Crit Care Med* 1994;**150**:956–61.
30. Prieto L, Gutierrez V, Uixera S, et al. Effect of cigarette smoking on airway responsiveness to adenosine 5′-monophosphate in subjects with allergic rhinitis. *Chest* 2003;**123**(4):993–7.
31. Willemse BW, ten Hacken NH, Rutgers B, et al. Smoking cessation improves both direct and indirect airway hyperresponsiveness in COPD. *Eur Respir J* 2004;**24**(3):391–6.
32. Polosa R. Adenosine-receptor subtypes: their relevance to adenosine-mediated responses in asthma and chronic obstructive pulmonary disease. *Eur Respir J* 2002;**20**(2):488–96.
33. Amin K, Ekberg-Jansson A, Lofdahl CG, et al. Relationship between inflammatory cells and structural changes in the lungs of asymptomatic and never smokers: a biopsy study. *Thorax* 2003;**58**(2):135–42.
34. Ekberg-Jansson A, Amin K, Bake B, et al. Bronchial mucosal mast cells in asymptomatic smokers relation to structure, lung function and emphysema. *Respir Med* 2005;**99**(1):75–83.
35. Thomas PS, Schreck RE, Lazarus SC. Tobacco smoke releases performed mediators from canine mast cells and modulates prostaglandin production. *Am J Physiol* 1992;**263**(1 Part 1): L67–72.
36. Small-Howard A, Turner H. Exposure to tobacco-derived materials induces overproduction of secreted proteinases in mast cells. *Toxicol Appl Pharmacol* 2005;**204**(2):152–63.
37. Kalenderian R, Raju L, Roth W, et al. Elevated histamine and tryptase levels in smokers' bronchoalveolar lavage fluid. Do lung mast cells contribute to smokers' emphysema? *Chest* 1988;**94**(1):119–23.
38. Spicuzza L, Bonfiglio C, Polosa R. Research applications and implications of adenosine in diseased airways. *Trends Pharmacol Sci* 2003;**24**(8):409–13.
39. Varani K, Caramori G, Vincenzi F, et al. Alteration of adenosine receptors in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2006;**173**(4):398–406.
40. Marthan R, Roux E, Savineau JP. Human bronchial smooth muscle responsiveness after in vitro exposure to oxidizing pollutants. *Cell Biol Toxicol* 1996;**12**:245–9.
41. Omini C, Hernandez A, Zuccari G, et al. Passive cigarette smoke exposure induces airway hyperreactivity to histamine but not to acetylcholine in guinea-pigs. *Pulm Pharmacol* 1990;**3**(3): 145–50.
42. Berger P, Compton SJ, Molimard M, et al. Mast cell tryptase as a mediator of hyperresponsiveness in human isolated bronchi. *Clin Exp Allergy* 1999;**29**(6):804–12.
43. Amrani Y, Chen H, Panettieri Jr RA. Activation of tumor necrosis factor receptor 1 in airway smooth muscle: a potential pathway that modulates bronchial hyper-responsiveness in asthma? *Respir Res* 2000;**1**(1):49–53.
44. McFadden Jr ER, Linden DA. A reduction in maximum mid-expiratory flow rate. A spirographic manifestation of small airway disease. *Am J Med* 1972;**52**(6):725–37.
45. Ciprandi G, Cirillo I, Tosca MA, et al. Bronchial hyperreactivity and spirometric impairment in patients with perennial allergic rhinitis. *Int Arch Allergy Immunol* 2004;**133**:14–9.