

## Induced sputum in adolescents with severe stable asthma. Safety and the relationship of cell counts and eosinophil cationic protein to clinical severity

D.C. Grootendorst<sup>\*\*\*</sup>, J-W. van den Bos<sup>\*</sup>, J.J. Romeijn<sup>\*</sup>, M. Veselic-Charvat<sup>+</sup>, E.J. Duiverman<sup>‡</sup>, E.J.L.E. Vrijlandt<sup>#</sup>, P.J. Sterk<sup>\*\*</sup>, A.C. Roldaan<sup>\*</sup>

*Induced sputum in adolescents with severe stable asthma. Safety and the relationship of cell counts and eosinophil cationic protein to clinical severity. D.C. Grootendorst, J-W. van den Bos, J.J. Romeijn, M. Veselic-Charvat, E.J. Duiverman, E.J.L.E. Vrijlandt, P.J. Sterk, A.C. Roldaan. ©ERS Journals Ltd 1999.*

**ABSTRACT:** This study examined the safety of sputum induction and the relation between sputum cell counts and clinical parameters in adolescents with severe persistent asthma.

Within 5 days, induced sputum and reversibility in forced expiratory volume in one second (FEV<sub>1</sub>), quality of life, provocative concentration causing a 20% fall in FEV<sub>1</sub> (PC<sub>20</sub>) of adenosine monophosphate and histamine, exercise-induced bronchoconstriction, overall asthma severity index, and blood eosinophils were collected in 20 atopic adolescents with moderate-to-severe persistent asthma (12–18 yrs of age, FEV<sub>1</sub> 65–110% of predicted, on 500–2,000 µg inhaled steroids daily).

FEV<sub>1</sub> was reversible by 13.3±2.3% pred. After sputum induction, FEV<sub>1</sub> was still increased by 9.0±2.6% pred as compared to the pre-salbutamol baseline. Sputum contained, median (range): 12.4 (0.4–59.5)% squamous cells, 47.3 (6.8–84.0)% macrophages, 39.0 (4.6–84.8)% neutrophils, 4.8 (1.0–12.4)% lymphocytes, 0.4 (0–10.8)% eosinophils and 3.6 (0–23.4)% bronchial epithelial cells. Sputum eosinophils showed a trend towards a significant association with the overall asthma severity index ( $r=0.46$ ,  $p=0.06$ ) and correlated inversely with baseline FEV<sub>1</sub> ( $r=-0.51$ ,  $p=0.03$ ).

In conclusion, sputum can be induced safely in adolescents with moderate-to-severe persistent asthma, if pretreated with  $\beta_2$ -agonists. Despite relatively low sputum eosinophil counts in these patients on inhaled steroids, the association of eosinophil numbers with baseline forced expiratory volume in one second and asthma severity index favours a role of induced sputum in monitoring adolescents with severe asthma.

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<sup>\*</sup>Dutch Asthma Centre, Davos, Switzerland. Depts of <sup>\*\*</sup>Pulmonology and <sup>+</sup>Pathology, Leiden University Medical Centre, Leiden, the Netherlands. <sup>‡</sup>Juliana Children's Hospital, The Hague, the Netherlands. <sup>#</sup>Medisch Spectrum Twente Hospital, Enschede, The Netherlands.

Correspondence: D.C. Grootendorst  
Lung Function Laboratory C2-P  
Dept of Pulmonology  
Leiden University Medical Centre  
P.O. Box 9600  
NL-2300 RC Leiden  
The Netherlands  
Fax: 31 715154691

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Asthma is a chronic disease, characterized by ongoing inflammation in the airways [1]. Studies using bronchial biopsies and bronchoalveolar lavage have demonstrated increased infiltration of eosinophils, mast cells and lymphocytes in the airways of subjects with asthma [2]. However, bronchoscopy is an invasive procedure, especially in children, and use is therefore restricted to adult patients with mild forms of asthma [3].

Induced sputum is currently considered to be a noninvasive alternative to bronchoscopy to examine airways inflammation. Induced sputum has been shown to yield reproducible data with regard to cellular and soluble markers of inflammation in asthmatic and healthy subjects as well as in smokers with chronic nonobstructive bronchitis [4, 5]. In subjects with asthma, induced sputum is characterized by increased numbers of eosinophils and mast cells as compared to sputum from healthy subjects [6, 7]. Naturally occurring acute exacerbations of asthma are associated with further increases of eosinophil [8] or neutrophil numbers [9]. In addition, eosinophil and neutrophil counts are elevated in induced sputum 24 h after an allergen challenge [10, 11], while treatment of asthmatic subjects with oral or inhaled corticosteroids (ICS) decreases the number of sputum eosinophils [12, 13].

When considering the relationship between sputum and airway physiology, it has been demonstrated that eosinophils and eosinophil cationic protein (ECP) in sputum are correlated with the degree of airway obstruction and symptom scores [6, 14, 15]. In addition, eosinophils in sputum are associated with bronchial hyperresponsiveness (BHR) when measured by methacholine [14] or histamine [15], but not by hypertonic saline [16]. Moreover, it has been shown in subjects with asthma that eosinophil numbers in sputum reflect those in bronchial washings, bronchoalveolar lavage fluid, and bronchial biopsies [17, 18]. These studies support the view that induction of sputum can be used to monitor airway inflammation in subjects with asthma.

While its noninvasive properties make it a potentially very useful tool to study airway inflammation in children and adolescents with asthma, very few studies on induced sputum have been conducted in this group. Pin *et al.* [19] have shown that in children with symptomatic mild asthma, sputum eosinophils are increased as compared to healthy children and children with asymptomatic asthma, with no statistical difference in sputum eosinophils between the latter two groups. In children with an acute exacerbation of asthma, induced sputum is characterized

by increased numbers of eosinophils, neutrophils and mast cells, which decrease again with resolution of the exacerbation [20]. So far, studies on induced sputum in relatively severe asthmatic children are lacking.

The aim of this study was to examine the safety of sputum induction in atopic adolescents with moderate-to-severe persistent asthma, and, secondly, to examine whether cell numbers in induced sputum are related to clinical parameters in this group of patients. To this end, 20 atopic asthmatic adolescents were examined in a cross-sectional study. Sputum was induced by inhalation of hypertonic saline in these atopic asthmatics. Differential cell counts obtained from whole processed sputum samples were related to diary card outcomes, quality-of-life scores, degree and reversibility of airways obstruction, BHR to different stimuli, markers of inflammation in blood and an overall index of asthma severity.

## Methods

### Patients

Twenty nonsmoking, atopic adolescents with asthma, optimally treated with moderate-to-very-high dose ICS [21], were invited to participate in this study (12 male, mean $\pm$ SEM age 14.9 $\pm$ 0.4 yrs (table 1)). The mean $\pm$ SEM dose of ICS in this group of patients was 1,295 $\pm$ 113.7  $\mu$ g of fluticasone (n=17), budesonide (n=2) or beclomethasone (n=1) daily (range 500–2,000  $\mu$ g). Based on these levels

of inhaled steroids, patients were characterized as having moderate or severe persistent asthma [22]. In addition to ICS, long-acting  $\beta_2$ -agonists were used by 17 of the patients, while all of them used short-acting  $\beta_2$ -agonists for relief of symptoms. All patients had to have a forced expiratory volume in one second (FEV<sub>1</sub>) >60% of predicted [23, 24], BHR to inhaled histamine as shown by a provocative concentration causing 20% fall in FEV<sub>1</sub> (PC<sub>20</sub>) of  $\leq$ 8 mg·mL<sup>-1</sup> [25], and atopy as indicated by a positive radioallergosorbent test (RAST; score >2). None of the patients had had symptoms of a respiratory tract infection within the four weeks prior to the study.

### Design

All patients attended the laboratory on three consecutive days. At the first visit, blood was drawn and the paediatric asthma quality-of-life questionnaire (PAQLQ) was completed. BHR to adenosine monophosphate (AMP) was measured in the morning, followed 4 h after spontaneous recovery by a bronchial provocation with histamine. A standardized exercise challenge was performed on day 2. Reversibility of FEV<sub>1</sub> to salbutamol was measured prior to sputum induction at the third visit. All patients were instructed to record morning and evening peak expiratory flow (PEF) rates and day- and night-time symptoms in a diary card for a 5-day period, commencing 2 days before the first challenge test until the day of sputum induction. Theophyllines, nedocromil and long-acting  $\beta_2$ -agonists

Table 1. – Subject characteristics

Subj.	Age yrs	FEV <sub>1</sub> % pred	Revers % pred	PC <sub>20</sub> AMP mg·mL <sup>-1</sup> *	PC <sub>20</sub> HIST mg·mL <sup>-1</sup> *	EIB max% fall	PEF <sub>m</sub> %pred	PEF <sub>v</sub> amp %mean	CSS	ASI	QoL	Blood eos. ×10 <sup>6</sup> ·L <sup>-1</sup>	% Sputum eos. <sup>†</sup>	Medication
1	15	60.5	20.2	6.4	0.13	25.0	66.0	1.3	20	10	5.9	395	5.8	1000 F, S
2	17	61.5	24.4	19.2	0.22	20.4	51.4	16.4	24	11	5.0	1316	5.6	1500 F, Fo
3	17	66.3	32.0	0.08	0.015	60.0	75.5	12.7	37	12	4.0	472	3.2	1000 F, 600 T, S
4	17	72.1	12.2	51.1	1.05	15.2	91.1	12.4	25	7	6.4	291	0	1600 B
5	15	72.7	12.8	19.6	0.21	16.3	68.7	7.4	26	10	5.7	700	0	1500 F
6	14	72.8	8.8	2.7	0.08	45.7	NA	NA	NA	ND	4.3	1342	–	2000 F
7	15	82.0	8.6	160.0	1.3	5.6	83.0	8.1	25	6	6.5	322	0.4	1000 F, S
8	16	83.2	15.1	6.1	0.015	18.6	83.5	8.4	11	8	4.3	503	0.4	1000 F, S
9	18	85.0	6.3	126.4	0.25	8.8	92.2	7.2	6	7	5.4	31	1.6	2000 F, S
10	17	86.5	26.5	0.08	0.015	51.5	74.0	12.8	19	9	4.8	222	0	2000 F, S
11	13	89.8	7.5	28.3	0.22	15.5	90.8	13.9	20	8	5.5	625	2.8	1500 F, S
12	14	90.1	15.6	2.6	0.17	31.9	67.6	4.1	79	11	2.7	1045	10.8	1000 F, S
13	15	90.4	2.2	80.0	1.41	NP	72.5	3.4	NA	ND	4.0	134	5.8	2000 F, 60 P, S
14	14	93.0	25.6	170.4	0.04	27.6	64.4	18.9	4	7	3.3	855	0.2	1000 F, S
15	12	93.8	0	224.9	0.95	7.9	86.6	10.4	5	5	6.2	300	0	500 F, S
16	13	94.0	14.6	25.5	0.14	9.1	78.4	6.6	5	6	6.6	272	5.0	1000 BDP, 8 N, S
17	12	96.5	2.8	10.9	0.015	23.9	68.3	5.9	11	9	4.6	200	0	800 B, S
18	14	99.2	4.8	27.2	2.7	1.7	64.4	17.9	32	8	4.1	131	2.8	2000 F, 8 N, S
19	15	103.1	13.0	17.3	1.2	4.2	NA	NA	NA	ND	5.9	191	0	500 F, S
20	15	115.9	0	0.9	0.21	24.5	82.6	11.4	24	9	3.1	681	0.6	1000 F, S
Mean (SEM)	14.9 (0.4)	85.4 (3.2)	13.3 (2.3)	12.2 (0.7)	0.19 (0.5)	21.8 (3.7)	75.6 (2.7)	10.0 (1.2)	22.6 (4.5)	8.4 (0.5)	4.9 (0.3)	322.0 (31.0–1316)	0.4 (0–10.8)	1295 (113.7)

Subj.: Subject; Revers: reversibility in forced expiratory volume in one second (FEV<sub>1</sub>), expressed as % pred; PC<sub>20</sub>AMP: provocative concentration of adenosine monophosphate causing a 20% fall in FEV<sub>1</sub>; PC<sub>20</sub>HIST: provocative concentration of histamine causing a 20% fall in FEV<sub>1</sub>; EIB: exercise-induced bronchoconstriction, expressed as maximal % fall in FEV<sub>1</sub>; PEF<sub>m</sub>: mean minimum morning peak expiratory flow, expressed as % pred; PEF<sub>v</sub>: peak expiratory flow variability, expressed as amplitude % mean; CSS: cumulative symptom score; ASI: asthma severity index; QoL: quality-of-life score; eos.: eosinophils. \*: Geometric mean, SD in doubling doses; †: median (range). NA: data not available; ND: not determined due to incomplete diary cards; NP: not performed; F: fluticasone; B: budesonide; BDP: beclomethasone; T: theophylline; P: prednisone; N: nedocromil; S: salmeterol; Fo: formoterol. –: no sputum sample obtained during sputum induction.

were discontinued from 48 h before the first challenge until after sputum induction at the third visit. Short-acting  $\beta_2$ -agonists were withheld for 8 h before each challenge test. In addition, patients were asked to refrain from caffeine-containing beverages in the 4 h preceding challenges. The study was approved by the ethical committees of the participating hospitals. Informed consent was given by the adolescents and their guardians.

### *Quality of life*

To assess the influence of asthma on day-to-day functioning (when the patients are using all their medication), the patients filled in the PAQLQ in the week prior to the study visits [26]. The PAQLQ contains five questions on activity limitation, 10 questions on symptoms and eight questions on emotional function. For each of the questions of the PAQLQ, patients were asked to recall which impairment they had experienced during the previous week. Response options were recorded on a 7-point scale where 1 indicates maximum impairment and 7 indicates no impairment. Results were expressed as mean score per domain and as the mean of all questions [26].

### *Diary cards*

Diary cards were completed during the period that the patients were refraining from their long-acting bronchodilators, *i.e.* from 2 days before the first challenge test until the day of sputum induction. The best of three PEF measurements, as obtained with a mini-Wright peak flow meter, was recorded every morning and evening, before medication usage. Severity of daytime dyspnoea, cough, sputum production and limitation in activities, and nocturnal dyspnoea and cough were registered on a scale ranging 0 (no symptoms) to 3. Diary cards were used to calculate the following parameters. The minimum morning PEF (PEF<sub>min</sub>) was determined as the lowest PEF in the study period and expressed as % pred [23]. Variation in PEF (PEF<sub>var</sub>) was calculated as diurnal variation (highest minus lowest value as a percentage of the mean) averaged over the study period (amplitude % mean). Scores for all symptoms during the 5-day period were added to yield a cumulative symptom score (CSS) ranging 0–90.

### *Spirometry and challenge tests*

Flow–volume curves and FEV<sub>1</sub> were recorded according to recommendations [24], using a Masterlab pneumotachograph (Jaeger, Würzburg, Germany) or a wet spirometer (Pulmonet3; Sensormedics, Bilthoven, the Netherlands). Results were compared to reference values for children and adolescents of QUANJER *et al.* [23].

Bronchial responsiveness to AMP (Merck, St Louis, MO, USA) in saline and to histamine diphosphate in phosphate-buffered saline were measured using the standardized 2 min tidal breathing method [25]. AMP and histamine solutions were administered to the patient in serial doubling concentrations ranging 0.15–320 mg AMP·mL<sup>-1</sup> and 0.03–8 mg histamine·mL<sup>-1</sup>, using a DeVilbiss 646 nebulizer (DeVilbiss Co., Somerset, PA, USA). With the method used, the normal ranges for AMP and histamine were >320 mg·mL<sup>-1</sup> and >8 mg·mL<sup>-1</sup>, respectively [25, 27]. After the last dose of AMP or histamine, spontaneous recovery of FEV<sub>1</sub> was measured at 3, 5, 7, 10 and 15 min. If

FEV<sub>1</sub> was not within 5% of baseline, measurements of FEV<sub>1</sub> were continued at 20, 30, 45 and 60 min, until FEV<sub>1</sub> was within 5% of baseline. PC<sub>20</sub> was calculated by linear interpolation of the last two points of the log concentration–response curves.

A standardized exercise challenge, with inhalation of compressed dry air (20°C, relative humidity <15%) was performed on a bicycle ergometer (ER900; Jaeger or Sensormedics, Bilthoven, the Netherlands) according to recommendations [25]. The work intensity was gradually increased for each of the subjects to achieve a minute ventilation between 40 and 60% of their predicted maximal voluntary ventilation (FEV<sub>1</sub> × 35) during the first 2 min of exercise. At this work intensity level, subjects exercised for 4 min. During exercise, ventilation was measured by a V<sub>max</sub> (Sensormedics spirometer). FEV<sub>1</sub> after exercise was measured regularly, using the time schedule as following the AMP or histamine challenge. The response to exercise was expressed as the maximal percentage fall in FEV<sub>1</sub> from baseline value.

### *Sputum induction*

Prior to sputum induction, FEV<sub>1</sub> was measured before and 15 min after inhalation of 200 µg salbutamol by a metered-dose inhaler (MDI) connected to an aerosol chamber, which was administered for safety reasons. The reversibility in FEV<sub>1</sub> was calculated as the difference between post- and prebronchodilator FEV<sub>1</sub>, expressed as % pred. Sputum was then induced by inhalation of hypertonic saline (4.5%) aerosols [4, 17]. Sodium chloride aerosols were generated at room temperature by a DeVilbiss Ultraneb 2000 ultrasonic nebulizer at maximal output setting (2.5 mL·min<sup>-1</sup>). The generated aerosols have a particle size with a mass median aerodynamic diameter of 4.5 µm [28]. Patients inhaled saline aerosols in periods of 5 min for a maximum of 15 min. Every 5 min, patients were asked to rinse their mouth and throat with water and to expectorate sputum into a clean plastic container. Sputum induction was discontinued if an adequate amount of sputum was produced (at least 0.5–1 g), if the 15 min of inhalation was completed or if the patient experienced any discomfort. After completion of the procedure and whenever the subject felt uncomfortable, FEV<sub>1</sub> was measured again. When FEV<sub>1</sub> fell by >20% from baseline during or after sputum induction, a final dose of 200 µg salbutamol was administered using a pressurized MDI (pMDI).

### *Sputum processing*

Whole sputum samples (sputum unseparated from saliva) were processed according to a published protocol [4, 17] in one centre within 2–4 h of sputum induction. In brief, a volume of 0.1% dithiothreitol (Sputolysin; Calbiochem, La Jolla, CA, USA), equal to the volume of the whole sputum sample, was added. Samples were gently mixed using a wide bore pipette, and placed in a shaking water bath at 37°C for 15 min to ensure complete homogenization. Homogenized sputum was centrifuged at 350 × g for 10 min. Supernatant was aspirated and stored at -20°C pending analysis. Cell pellets were resuspended in phosphate-buffered saline and filtered through a nylon mesh (pore size 48 µm; Thompson, Ontario, Canada). Cell viability and total cell count were then established by

trypan blue exclusion using a haemocytometer. Cytospin slides were prepared by cytocentrifugation for 3 min at  $250 \times g$ , with a cell suspension containing  $0.5\text{--}1.0 \times 10^6$  cells·mL<sup>-1</sup> (Shandon cytocentrifuge 3; Shandon Southern Instruments, Sewickley, PA, USA). Differential cell counts were made by a qualified cytologist, on coded May–Grünwald–Giemsa stained cytopins and expressed as percentage of 500 cells, excluding squamous cells. Furthermore, absolute cell counts were calculated as the number of cells·mL<sup>-1</sup>. Sputum samples containing >80% squamous cells were excluded from analysis because of poor cytopsin quality [4].

### Blood eosinophils and ECP

Peripheral blood eosinophils were counted by an automated counter and expressed as total cells·L<sup>-1</sup>. Serum was separated after allowing the samples to stand for 1 h and centrifugation at  $925 \times g$  for 10 min. ECP in sputum supernatants and serum was determined by radioimmunoassay (Pharmacia, Uppsala, Sweden) and expressed as ng·mL<sup>-1</sup>.

### Overall asthma severity index

The above measurements were used to calculate an overall asthma severity index. This was based on cumulative symptom score (CSS), medication usage, baseline FEV<sub>1</sub> and PC<sub>20</sub> of histamine, each of those arbitrarily being divided into five categories [21, 29] (table 2). The overall asthma severity index was calculated as the sum of the five categories from the four variables, and could thus range from 0 to 16.

### Analysis

Baseline FEV<sub>1</sub> was calculated as the mean of the morning FEV<sub>1</sub> on the three consecutive study days. Change in FEV<sub>1</sub> after sputum induction was calculated as the difference between FEV<sub>1</sub> recorded after sputum induction and pre-salbutamol FEV<sub>1</sub>, expressed as % pred. Owing to their highly skewed distribution, data on PC<sub>20</sub> of AMP and PC<sub>20</sub> of histamine, percentage eosinophils, neutrophils and

bronchial epithelial cells in sputum, eosinophil counts in blood, ECP levels in serum and sputum, and CSS, were log-transformed before analysis. Since the non-normally distributed variables contained zeros, the value of one was added to all data prior to log-transformation. Data are presented as mean±SEM or, in cases of non-normal distribution, as median (range). Correlations between sputum eosinophils, neutrophils and ECP on the one hand and clinical parameters on the other were tested using the parametric technique of Pearson. Analyses were performed with the Statistical Package for Social Sciences (SPSS-PC+; SPSS, Chicago, IL, USA). A p-value <0.05 was considered significant.

## Results

One patient had a progressive fall in FEV<sub>1</sub> (>20 % pred) when measuring baseline spirometry, and PC<sub>20</sub> values for AMP and histamine were arbitrarily set at 0.15 and 0.015 mg·mL<sup>-1</sup>, respectively. Exercise could not be performed in one patient, whereas it resulted in a fall in FEV<sub>1</sub> of >60% from baseline requiring rescue medication in one other patient, so that recovery in FEV<sub>1</sub> could not be measured. Diary cards were completed by 17 patients. Clinical and lung function data are shown in table 1, and sputum and blood contents in table 3. There was a close correlation between the overall asthma severity index and PC<sub>20</sub> of AMP ( $r=-0.70$ ,  $p<0.01$ ), exercise-induced bronchoconstriction (EIB) expressed as the maximum % fall in FEV<sub>1</sub> ( $r=0.67$ ,  $p<0.01$ ), % eosinophils in blood ( $r=0.54$ ,  $p<0.05$ ), PEFmin ( $r=-0.55$ ,  $p<0.05$ ) and paediatric quality-of-life score ( $r=-0.51$ ,  $p<0.05$ ).

### Lung function during and after sputum induction

Inhalation of hypertonic saline was well tolerated by all adolescents. The mean±SEM duration of sputum induction was  $11.7\pm0.8$  min. The mean and individual FEV<sub>1</sub> before and after salbutamol pretreatment and after sputum induction are shown in figure 1. The individual changes in FEV<sub>1</sub> after sputum induction as compared to the pre-salbutamol baseline value ranged between an increase of 30.2 % pred and a decrease of 12.6% pred. The percentage change in FEV<sub>1</sub> after sputum induction was significantly correlated with pre-salbutamol baseline FEV<sub>1</sub> in % pred ( $r=-0.57$ ,  $p<0.01$ ), reversibility in FEV<sub>1</sub> ( $r=$

Table 2. – Categories for asthma medication usage [21], cumulative symptom score (CSS), baseline forced expiratory volume in one second (FEV<sub>1</sub>) and provocative concentration of histamine causing a 20% fall in FEV<sub>1</sub> (PC<sub>20</sub>) to calculate the overall asthma severity index

Cate- gory	Medication	CSS*	FEV <sub>1</sub> % pred	PC <sub>20</sub> mg·mL <sup>-1</sup>
0	β <sub>2</sub> -agonists only	0–5	>80	>8
1	Low dose ICS <sup>†</sup>	6–20	70–80	4–8
2	Moderate <sup>‡</sup> –high dose ICS <sup>§</sup>	21–40	60–70	1–4
3	High–very high dose ICS <sup>#</sup> ± oral steroids	41–60	50–60	0.25–1
4	Very high dose ICS + oral steroids + additional therapy	>60	<50	<0.25

\*: CSS obtained from the diary cards; <sup>†</sup>: low dose inhaled corticosteroids (ICS), beclomethasone dipropionate (BDP) ≤500 µg, fluticasone (FP) ≤250 µg, budesonide (Bu) ≤400 µg daily; <sup>‡</sup>: moderate dose ICS, BDP 500–1,000 µg, FP 250–500 µg, Bu 400–800 µg daily; <sup>§</sup>: high dose ICS, BDP 1,000–2,000 µg, FP 500–1,000 µg, Bu 800–1,600 µg daily; <sup>#</sup>: very high dose ICS, FP 1,000–2,000 µg, Bu 1,600–3,200 µg daily.

Table 3. – Total cell count, viability, differential cell count and eosinophil cationic protein (ECP) in induced sputum, and eosinophils and ECP in blood

	Induced sputum	Blood
Total cell count ×10 <sup>6</sup> ·mL <sup>-1</sup>	9.0 (0.5–71.0)	
Viability %	85.3 (52.9–98.2)	
% squamous cells	12.4 (0.4–59.5)	
% macrophages	47.3 (6.8–84.0)	
% neutrophils	39.0 (4.6–84.8)	
% eosinophils*	0.4 (0–10.8)	358.5 (31.0–1342.0)
% lymphocytes	4.8 (1.0–12.4)	
% bronchial epithelial cells	3.6 (0–23.4)	
ECP ng·mL <sup>-1</sup>	193.4 (4.0–1296)	15.5 (2.0–135.0)

Data are presented as median (range). \*: Eosinophils in blood are presented as number of cells ×10<sup>6</sup>·mL<sup>-1</sup>.

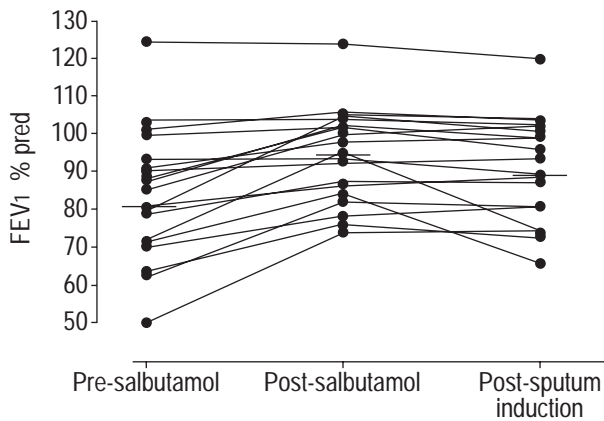


Fig. 1. – Forced expiratory volume in one second (FEV<sub>1</sub>) as measured before and after salbutamol pretreatment and after sputum induction. Prior to sputum induction, baseline FEV<sub>1</sub> increased significantly from mean $\pm$ SEM 83.0 $\pm$ 3.9 % pred to 95.3 $\pm$ 3.1 % pred ( $p<0.001$ ) after salbutamol. After sputum induction, FEV<sub>1</sub> was 91.3 $\pm$ 3.3 % pred, which was significantly better as compared to pre-salbutamol baseline ( $p=0.001$ ). Horizontal bars represent the mean of all samples.

0.74,  $p<0.01$ ) and PC<sub>20</sub> of histamine ( $r=-0.49$ ,  $p<0.05$ ), with a trend towards such a correlation with peripheral blood eosinophils ( $r=0.45$ ,  $p=0.053$ ).

Sputum induction did not have any bronchoconstrictive effects after the monitoring period since neither PEF rates on the evening, nor the morning following sputum induction, were different from PEF rates on the morning prior to sputum induction ( $p>0.1$ ).

#### Induced sputum samples

Sputum was induced successfully in 19 of the patients, whereas one patient was unable to cough up a sputum sample within the 15 min induction period. Mean and individual total cell counts, viability, % squamous cells, differential cell count and ECP levels in sputum supernatants are presented in table 3. Sputum differential cell counts and ECP did not correlate with lung function or airway hyperresponsiveness, or with the diary card outcomes or quality of life. However, there was a trend towards a correlation between % eosinophils in sputum and asthma severity index ( $r=0.46$ ,  $p=0.06$ ; fig. 2). In addition,

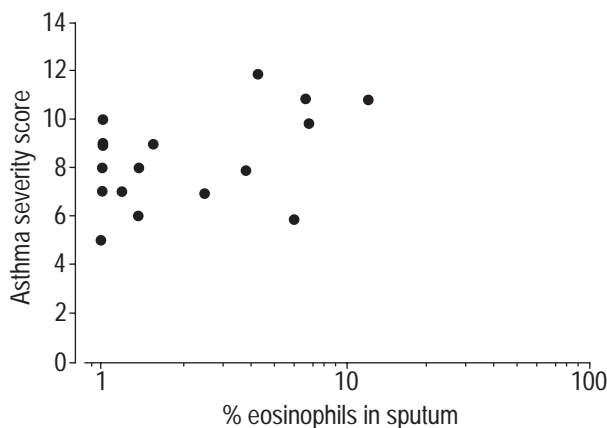


Fig. 2. – The relationship between the percentage sputum eosinophils and the asthma severity score. Eosinophils in induced sputum borderline significantly correlated with the asthma severity score ( $r=0.46$ ,  $p=0.06$ ).

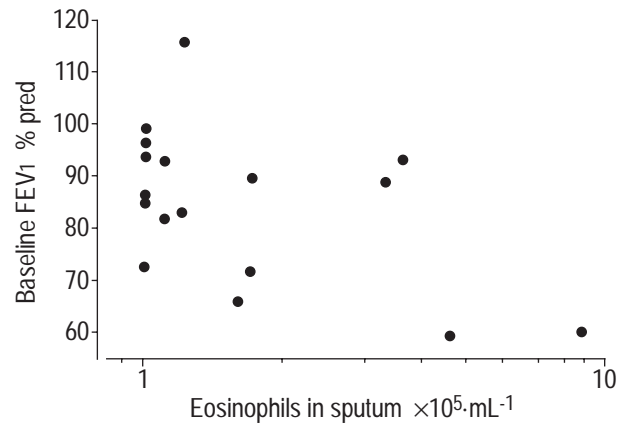


Fig. 3. – The relationship between absolute sputum eosinophil counts in induced sputum and baseline forced expiratory volume in one second (FEV<sub>1</sub>). Eosinophil numbers in induced sputum correlated inversely with baseline FEV<sub>1</sub> ( $r=-0.51$ ,  $p=0.03$ ).

absolute numbers of eosinophils in sputum correlated with baseline FEV<sub>1</sub> ( $r=-0.51$ ,  $p=0.047$ ; fig. 3). Eosinophils and neutrophils in sputum correlated with ECP levels in sputum supernatant ( $r=0.60$ ,  $p<0.01$  and  $r=0.68$ ,  $p<0.01$ , respectively). Sputum eosinophils were weakly associated with blood eosinophil numbers ( $r=0.43$ ,  $p=0.09$ ). Furthermore, there was a trend towards correlation between sputum and serum ECP levels ( $r=0.43$ ,  $p=0.06$ ).

#### Discussion

This study shows that sputum can be induced safely and successfully in adolescents with moderate-to-severe persistent asthma who are currently treated with high-dose ICS. The sputum induction procedure did not result in clinically significant bronchoconstriction after pretreatment with 200  $\mu$ g inhaled salbutamol. Sputum samples of these steroid-treated patients contained few % eosinophils which were, nevertheless, correlated with ECP levels in sputum supernatant and baseline FEV<sub>1</sub>, with a similar trend for asthma severity index. These results suggest that induction of sputum by inhalation of 4.5% hypertonic saline is an easily applicable, noninvasive and safe method to study airway inflammation in patients with moderate-to-severe persistent asthma, aged between 12 and 18 yrs. Induced sputum samples may yield additional information in the monitoring of these patients.

To the authors' knowledge, this is the first study examining the safety and validity of sputum induction by inhalation of hypertonic saline aerosols in atopic asthmatic teenagers with moderate-to-severe persistent asthma. In 19 of these patients, <15 min inhalation was enough to yield a sputum sample. When patients were pretreated with salbutamol, FEV<sub>1</sub> at the end of sputum induction was still increased as compared to pre-salbutamol baseline in the majority of patients. Interestingly, none of the patients experienced a drop in FEV<sub>1</sub> after sputum induction >13% pred and such a drop appeared to develop only in those patients with a relatively high baseline FEV<sub>1</sub> and with the smallest response to inhaled salbutamol. Indeed, the change in FEV<sub>1</sub> after sputum induction was inversely related to pre-salbutamol baseline FEV<sub>1</sub> and PC<sub>20</sub> histamine, and positively correlated with reversibility in FEV<sub>1</sub>. This indicates that patients with the greatest reversibility in

airways obstruction are best protected by  $\beta_2$ -agonists against potential bronchoconstrictive effects of inhaling hypertonic saline during sputum induction. These results confirm and extend those of WONG *et al.* [30] although these authors' also observed a relationship between the change in FEV<sub>1</sub> after sputum induction and sputum eosinophils, which could not be demonstrated in the present patients.

The present results were obtained using standardized and validated techniques. Firstly, patients with asthma, using moderate-to-very-high dose ICS regularly to control the disease [21], were selected from the outpatient departments of several hospitals. All patients were seen at 6-month intervals by their treating specialist. Patients were treated for their asthma according to recent guidelines and ICS dosages were reduced stepwise to the lowest dose at which symptoms were controlled [31]. Patients were thus treated by an optimal dose of ICS. Secondly, BHR was measured to an indirect stimulus (AMP), as well as to a direct stimulus (histamine), on the same day, in order to minimize the number of study days. This was justified by the observation that BHR to histamine measured after an AMP challenge was not different from before [32]. Thirdly, in order to validate the safety of sputum induction, FEV<sub>1</sub> measured directly after completion of the sputum induction procedure was compared to the pre-salbutamol baseline value instead of to the post-salbutamol FEV<sub>1</sub> as carried out by WONG *et al.* [30]. The current authors believe that this approach is clinically more relevant because any worsening in FEV<sub>1</sub> after the procedure is being related to the patients real baseline FEV<sub>1</sub>. Fourthly, the method used in this study to induce and process sputum has been validated extensively [4]. Fifthly, the PAQLQ is a recently developed questionnaire to establish asthma-related quality of life in children aged between 7 and 17 yrs [26]. The questionnaire is reproducible in Canadian children and teenagers with stable asthma and is able to detect changes in patients whose asthma improves or deteriorates [26]. The officially translated Dutch version of the PAQLQ was used in the present study. Although validation of the Dutch version of the questionnaire in a Dutch population has not been published yet, it can be assumed that cultural differences between Canadian and Dutch children are relatively small and unlikely to interfere with validity or responsiveness of the questionnaire. Finally, a score for overall asthma severity was calculated based on current treatment level, level of baseline FEV<sub>1</sub>, level of symptoms and level of hyperresponsiveness to histamine (table 2) [21]. This apparently yielded a representative score for severity of asthma since it correlated with the bronchoconstrictive response to exercise, and peripheral blood and sputum eosinophils, while it correlated inversely with BHR to AMP, PEF<sub>min</sub> and quality of life.

Percentages of sputum eosinophils in this study appeared to be low, in particular when taking into account the level of symptoms and BHR to histamine in these patients and when comparing the sputum eosinophils numbers to those in healthy children (median % sputum eosinophils 0.15) [19]. It has been shown by PIACENTINI *et al.* [33] that induced sputum in asthmatic children contains 10.8% eosinophils. However, most of these children were treated with low-to-moderate doses of inhaled beclomethasone, which was discontinued in the week before collection of induced sputum. Since treatment with inhaled steroids

results in a decrease in sputum eosinophils [13], withdrawal of asthma medication in the latter studies might have resulted in an increase in eosinophil numbers in sputum. The relatively low eosinophil counts in the present study may suggest that the adolescents were adequately treated with inhaled steroids and were compliant to their therapy, resulting in suppressed inflammation within the airway lumen. Indeed, sputum samples obtained from teenagers with mild stable asthma with or without symptoms, not requiring regular treatment, contains only 1.7% and 0.4% eosinophils, respectively [19]. It should be noted that absence of luminal inflammation in these patients with asthma using moderate-to-very-high dose ICS, does not necessarily mean that mucosal inflammation is equally suppressed. Although it has previously been shown that sputum eosinophils may reflect the number of tissue eosinophils, subjects with no eosinophils in sputum may still have a considerable variation in mucosal eosinophil number [17, 18].

Despite the relatively low eosinophil numbers in sputum, these cells were shown to be associated with baseline FEV<sub>1</sub> and asthma severity score, which is in accordance with previous studies [6, 14, 15]. In addition, it was demonstrated that eosinophils in sputum are related to ECP levels in sputum supernatants, suggesting activation of those cells within the airways. Surprisingly, such a correlation could also be demonstrated for sputum neutrophils. Recent *in vitro* experiments have shown that neutrophil-derived proteases such as elastase can cause eosinophil degranulation [34], which may explain the observed relation between sputum neutrophils and ECP.

What are the clinical implications of the present results? Induction of sputum by inhalation of hypertonic saline aerosols is a safe procedure in teenagers with moderate-to-severe persistent asthma, provided that they are pretreated with an adequate amount of inhaled bronchodilator. Sputum induction is apparently an easily applicable and well-tolerated method for studying airway inflammation in this age group of asthmatic patients. In this cross-sectional analysis, cell numbers in induced sputum appeared to be associated with the degree of airway obstruction, but not with bronchial hyperresponsiveness, diary card outcomes or quality of life. It is, therefore, likely that examining airway inflammation in induced sputum samples provides complementary information on top of symptoms and lung function, which may be useful in long-term monitoring of adolescent patients with severe asthma. Obviously, this needs to be examined in careful prospective studies.

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