Airway inflammatory response to ozone in subjects with different asthma severity

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ABSTRACT: The aim of this study was to evaluate whether ozone exposure induces a similar airway inflammatory response in subjects with different degrees of asthma severity.

Two groups of asthmatic subjects were studied: seven with intermittent mild asthma not requiring regular treatment (group A); and seven with persistent mild asthma requiring regular treatment with inhaled corticosteroids and long-acting β_2 -agonists (group B). All subjects were exposed, in a randomized cross-over design, to air or O_3 (0.26 parts per million (ppm) for 2 h with intermittent exercise); subjects in group B withdrew from regular treatment 72 h before each exposure. Before the exposure, and 1 and 2 h after the beginning of the exposure they performed a pulmonary function test, and a questionnaire was completed to obtain a total symptom score (TSS). Six hours after the end of the exposure, hypertonic saline (HS) sputum induction was conducted. Sputum cell percentages, eosinophil cationic protein (ECP) and interleukin (IL)-8 concentrations in the sputum supernatant were measured.

TSS significantly increased and forced vital capacity (FVC) and forced expiratory volume in one second (FEV₁) significantly decreased after O_3 exposure in comparison with air exposure in group A, whereas no changes were observed in group B except for a significant decrement of FEV₁ 2 h after the beginning of O_3 exposure. Sputum neutrophil percentage was significantly higher after O_3 exposure than after air exposure in both groups (Group A: 70.2% (28–87) versus 26.6% (8.6–73.2); Group B: 62.1% (25–82.4) versus 27.9% (14.4–54)). IL-8 was higher in sputum supernatant collected 6 h after O_3 exposure than after air, only in group A. No change due to O_3 has been found in sputum eosinophil percentage and ECP concentration in both groups.

In conclusion, the degree of airway response to a short-term exposure to ozone is different in subjects with asthma of different severity. The available data do not allow elucidation of whether this difference depends on the severity of the disease or on the regular anti-inflammatory treatment

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Epidemiological studies have shown that high ambient concentrations of ozone are strictly associated with the occurrence of acute respiratory symptoms and increased rates of hospital admission in adults and children [1, 2]. In laboratory studies it has been confirmed that short-term exposure to O_3 induces in both healthy and asthmatic subjects a significant decrement in forced vital capacity (FVC) and forced expiratory volume in one second (FEV₁), elicits a respiratory discomfort and an increased airway reactivity to methacholine and allergen [3–6]. The pathophysiological background of this airway response is a neutrophilic inflammation of the airways accompanied by an increased level of proteins and mediators as demonstrated by bronchoalveolar lavage (BAL) technique [7]. Although previous studies reported different results regarding the effects on pulmonary function due to O_3 exposure in normal and asthmatic subjects [4, 8], more recently, other studies have demonstrated that asthmatics have a greater inflammatory

response in BAL fluid after O_3 exposure [9, 10]. HILTERMANN *et al.* [11] showed that 0.4 parts per million (ppm) O_3 exposure induced a high sputum neutrophil recruitment in the airways in both normal and asthmatic subjects, the difference between healthy and asthmatics in the percentage of neutrophils being significant.

The aim of the present study was to compare the airway inflammatory response due to O_3 between two groups of asthmatic subjects with different degrees of asthma according to international guidelines [12]: group A, subjects with mild intermittent asthma, asymptomatic and without regular treatment; and group B, subjects with mild persistent asthma, requiring regular treatment to control asthma symptoms. To detect the inflammatory response of the airways, the technique of hypertonic saline (HS) induced sputum has been used. It is a reproducible, noninvasive and safe technique [13] and it has been proven to be useful for asthma diagnosis and for disease monitoring [14].

Subjects and methods

Subjects

Two groups of asthmatic subjects were studied: seven subjects with intermittent mild asthma not requiring regular treatment (group A); seven subjects with persistent mild asthma requiring regular treatment with inhaled corticosteroids (beclomethasone dipropionate 500 µg *b.i.d*) and long-acting β_2 -agonist (salmeterol 50 µg *b.i.d*) to control asthma symptoms (group B).

Diagnosis of asthma and assessment of asthma severity was performed according to Global Initiative for Asthma (GINA) criteria [12]. In particular, asthma was defined in the presence of a history of recurrent attacks of reversible dyspnoea with wheezing, and bronchial hyperreactivity to methacholine.

In order to establish the degree of asthma severity and the requirement for regular treatment, in a preliminary evaluation each subject recorded asthma symptoms and peak expiratory flow (PEF) measurements in the early morning and in the evening during a 1-week period, after withdrawal of regular treatment and using only inhaled salbutamol as rescue medication. PEF variability was expressed as mean value of the maximal amplitude (highest-lowest/daily mean) of each day of monitoring. The score of asthma symptoms (for each day: from 0 to 4 for nocturnal symptoms, and from 0 to 5 for diurnal symptoms) and the use of rescue salbutamol were expressed as weekly sum of the scores obtained in all days of monitoring. At the end of the week of PEF monitoring they measured FEV₁, FVC and bronchial hyperresponsiveness to methacholine.

The main clinical and functional findings of the two groups of subjects are reported in table 1. The study was approved by the Hospital Medical Ethic Committee, and

informed consent was obtained from all participants or their parents

Study protocol

All subjects were free from upper respiratory infection at least 6 weeks before the study. Patients in group B withdrew treatment 72 h before each study day.

Exposures were administered in a single-blind manner. All subjects were randomly exposed to O₃ (0.26±0.04 ppm) or filtered air (sham) for 2 h in a challenge chamber on two different days, at least 1 week apart (median (range): 14 (7, 28) days), while exercising on a cycloergometer at work load predetermined to produce a ventilation rate of 25 L·min⁻¹·m⁻² of body area surface. All of them attended the laboratory at 8:00 h on three different days. On day 1 they performed the cycloergometer work-load test to establish the target work load inducing a ventilation rate near 25 L·min⁻¹·m⁻². On days 2 and 3 they were exposed to O₃ or filtered air for 2 h. The sequence of exposure was: air-ozone in six subjects, ozone-air in eight subjects. Before the exposure, and 1 and 2 h after the beginning of the exposure they performed pulmonary function tests (PFT) using a computerized water-sealed bell spirometer (Biomedin, Padova, Italy) and a questionnaire was completed. Each subject was asked to grade from 0 (0=no symptom) to 4 (4=worst symptom) the severity of each symptom: cough, shortness of breath, tearing, eyes burning, throat and nose irritation, chest pain on deep inspiration, headache, dizziness, nausea, confusion, and sweating. A total symptom score (TSS) was computed for each subject as the sum of all single symptom scores. Six hours after the end of both chamber exposures, PFT and HS sputum induction were conducted.

Table 1. – Anthropometric and functional data of the 14 asthmatic subjects examined

Subject No.	Sex	Age yrs	Height cm	FEV1 %	FVC %	PD20 mg	MA %	ASS		RS puffs·week ⁻¹
								Night	Day	
Group A										
1	M	24	184	103	93	0.272	3	0	2	2
2	M	29	173	105	106	0.204	1	1	0	0
3	F	35	154	99	87	0.607	2	1	1	0
4	M	22	173	102	90	0.300	6	0	3	2
5	M	25	186	78	100	0.112	3	0	2	0
6	M	15	190	92	103	0.202	2	0	2	0
7	M	19	175	121	110	0.220	4	0	2	1
Mean±SD		24.0±6.5	176±11.9	100±13.1	98.4±8.6		3.6±1.5	0.3±0.5	2±0.8	0.7±0.9
GM						0.274				
Group B										
1	M	35	172	71	81	0.101	8	9	6	16
2	M	20	171	116	110	0.170	5	4	4	6
3	M	18	170	106	114	0.166	13	7	6	4
4	M	31	183	111	102	0.031	7	2	8	4
5	M	41	168	117	104	0.815	6	4	2	4
6	M	40	170	81	94	0.028	10	4	5	9
7	M	23	169	84	94	0.220	7	4	6	10
Mean±SD		29.7±9.4	171.8±5.1	98±18.8	99.8±11.7		8±2.7*	4.8±2.3*	5.2±1.9*	7.6±1.9*
GM						0.174				

FEV₁: forced expiratory volume in one second; FVC: forced vital capacity; PD20: provocative dose of methacholine causing a 20% fall in FEV₁ from baseline; MA: maximal amplitude; ASS: weekly asthma symptom score; RS: rescue salbutamol; GM: geometric mean; *: p<0.05, in comparison with group A.

Methods

Challenge chamber. The subjects were exposed for 120 min in a 9 m³ static challenge chamber made of glass and aluminium [15], while exercising on a stationary cycloergometer for 20 min every hour. Mean air temperature was 21±1°C and the relative humidity was 45±5%. Ozone was generated by a corona discharge O₃ generator (Rancon Instruments, Milan, Italy) connected to a cylinder of purified air. O₃ output into the chamber was 0.5 L·min⁻¹. An O₃ analyser (Photometric O₃ Analyser 400, Rancon Instruments) connected to the chamber by a tubing circuit, continuously monitored gas concentration in the chamber. Mean±SD O₃ concentration was 0.26±0.04 ppm. A fan in the chamber ensured adequate gas mixing and circulation.

Hypertonic saline inhalation test and sputum processing. HS solution was nebulized with an ultrasonic nebulizer (2.8 mL·min⁻¹ output; Sirius, Technomed, Firenze, Italy) and was inhaled for 5 min periods for up to 30 min. NaCl concentration was increased at 10-min intervals from 3 to 4 to 5%. Every 5 min after the start of nebulization, patients were asked to rinse their mouth and throat carefully in order to discard saliva, and to try to cough sputum into a clean container; then FEV₁ was measured. Nebulization was stopped after 30 min or when FEV₁ fell by ≥20% from baseline. The functional response to HS inhalation was expressed as maximum FEV₁ fall after HS inhalation (ΔFEV₁-HS%).

Sputum was processed as previously reported [16]. The whole sputum sample was diluted with an equal volume of 0.1% dithiotreitol (Sputasol; Unipath, Basingstoke, UK). The sample was incubated in a shaking bath at 37°C for 20 min, then gently mixed to further dissolve mucus plugs. At the end of incubation, sample was filtered through a 53 µm nylon gauze to remove debris. An aliquot of sputum sample was cytocentrifuged for 5 min (Cytospin; Shandon Scientific, Sewickley, PA, USA) and stained with Diff-Quik (Baxter Scientific Products, Miami, FL, USA). Two investigators, blinded to the subject's history, each first counted at least 500 cells on each sputum slide so as to obtain the squamous cell percentage as an indicator of saliva contamination. Cytospin slides on which 500 non-squamous cells could not be counted were considered unsatisfactory and discarded. At least 500 nonsquamous cells were then counted on satisfactory slides. All cell percentages were averaged to give the final values reported. Macrophage, lymphocyte, neutrophil, and eosinophil percentages are expressed as percentage of total inflammatory cells, excluding squamous cells. The remainder of the sputum sample was centrifuged at 450 × g for 10 min. The supernatant was collected and stored at -80°C for the analysis of soluble markers. The cell pellets were resuspended in normal saline for total cell counts with Turk staining and cell viability assessment by Trypan blue exclusion in a haemocytometer.

In the laboratory, this method gives a reasonably good reproducibility of inflammatory cell counts in sputum induced by HS. The reproducibility has been evaluated in 20 stable mild-to-moderate asthmatic subjects who repeated HS inhalation on two different days, a week apart, in a stable condition. The intraclass correlation coefficients (*r_i*) between two measurements were: +0.90 for macrophage,

+0.23 for lymphocyte, +0.88 for neutrophil, and +0.82 for eosinophil percentage [17].

Eosinophilic cationic protein level in sputum supernatant. This was measured by means of a specific radioimmunoassay (RIA; Pharmacia RIA, Uppsala, Sweden). This method has been proven highly specific and sensitive (lower detection limit: <2 µg·L⁻¹) with high intra- and interassay reproducibility.

Interleukin-8 level in sputum supernatant. Antigenic interleukin (IL)-8 levels in sputum supernatants were quantified using a sandwich enzyme-linked immunosorbent assay (ELISA). Briefly, undiluted and serial dilutions of the sputum supernatants and standard human recombinant IL-8 (R&D, Minneapolis, MN, USA) were placed into microtitre plates (Immulon, Chantilly, VA, USA) precoated with goat anti-human IL-8 antibody (R&D) diluted 1:2,000. After incubation for 1.5 h, rabbit anti-human IL-8 antibody (Upstate Biotechnology, Lake Placid, NY, USA) diluted 1:3,000 was added. After additional incubation for 1.5 h, peroxidase conjugated goat anti-rabbit antibody (ICN Biomedicals, Costa Mesa, CA, USA) was added at a 1:2,000 dilution. *o*-Phenylenediamine (OPD) in a 0.01% H₂O₂ solution was used as the substrate. The absorbance was measured at 492 nm. The lower limit of IL-8 detectable by this assay is 10 pM. All samples were assayed in duplicate, and the mean value was considered.

Statistical analysis

FVC and FEV₁ (as % of the predicted value), TSS, maximum %FEV₁ fall and duration of HS inhalation are expressed as mean±SD. Differential cell percentages in induced sputum, eosinophil cationic protein (ECP) and IL-8 levels in the supernatant of induced sputum are expressed as median and range, while total inflammatory cells were examined as mean±SD.

Paired t-tests were used to compare FVC and FEV₁ values, and TSS pre- and postexposure to air with the same values obtained after O₃ exposure. Analysis of variance (ANOVA) tests were used to compare FVC, FEV₁ and TSS at the different time-points after both air and O₃ exposures. Cell percentages, ECP and IL-8 levels in induced sputum were compared between air and O₃ exposure by using nonparametric Wilcoxon test.

A comparison of functional and biological data between two groups, both after air and after O₃ exposure, was performed by ANOVA test or by Mann-Whitney test, as appropriate.

Results

Clinical and functional evaluation

Pre-exposure values of FVC, FEV₁ and TSS were not significantly different between air and O₃ exposures in both groups (table 2). Both FVC and FEV₁ measured at the end of 1 and 2-h exposure and 6 h after the end of O₃ exposure were significantly lower than FVC and FEV₁ measured at the same time-points after air exposure in

Table 2. — Mean $\pm$ SD values of forced expiratory volume in one second (FEV₁), forced vital capacity (FVC), total symptom score (TSS) and Δ FEV₁-hypertonic saline (HS)% before and at different time-points after exposure to ozone and air

	Group A Mild intermittent		Group B Mild persistent	
	Ozone	Air	Ozone	Air
FEV ₁ pre-exposure %	91.0 $\pm$ 15	93.1 $\pm$ 14	96.0 $\pm$ 13	95.1 $\pm$ 14
FEV ₁ 1 h %	88.2 $\pm$ 7.1	96.7 $\pm$ 9.2*	93.3 $\pm$ 15	98.0 $\pm$ 14.7
FEV ₁ 2 h %	82.1 $\pm$ 12	98.6 $\pm$ 15*	92.8 $\pm$ 12	98.6 $\pm$ 11*
FEV ₁ 6 h %	89.7 $\pm$ 12	99.1 $\pm$ 12*	87.5 $\pm$ 16	96.0 $\pm$ 12.3
FVC pre-exposure %	93.4 $\pm$ 9.2	94.1 $\pm$ 9.9	96.0 $\pm$ 8.3	97.1 $\pm$ 9.3
FVC 1 h %	89.2 $\pm$ 7.2	94.4 $\pm$ 9.1*	97.1 $\pm$ 7.7	97.7 $\pm$ 7.7
FVC 2 h %	84.4 $\pm$ 10 [§]	97.5 $\pm$ 11*	94.6 $\pm$ 6.4	95.4 $\pm$ 8.2
FVC 6 h %	89.7 $\pm$ 8.2	95.6 $\pm$ 9.8*	99.7 $\pm$ 9.1	99.7 $\pm$ 8.2
TSS pre-exposure	3 $\pm$ 2.7	2.8 $\pm$ 2.8	1.4 $\pm$ 0.9	1.0 $\pm$ 1.4
TSS 1 h	7.3 $\pm$ 3.1 [§]	2.7 $\pm$ 2.6*	2.7 $\pm$ 1.6	1.5 $\pm$ 1.3
TSS 2 h	10 $\pm$ 5.5 [§]	4.8 $\pm$ 5.2*	5.1 $\pm$ 3 [§]	2.1 $\pm$ 0.2
Δ FEV ₁ -HS	33.1 $\pm$ 19.7	30 $\pm$ 12.8	23.3 $\pm$ 9.7	20.1 $\pm$ 8.9

Δ FEV₁-HS%: maximum FEV₁ fall after HS inhalation. *: $p < 0.05$, between air and ozone exposure (by paired t-test). [§]: $p < 0.05$, with respect to pre-exposure (by ANOVA test).

mild intermittent asthmatic subjects. In mild persistent asthmatic subjects there were no significant differences for FVC and FEV₁ between post-air and post-O₃ exposure, except at 2 h for FEV₁ (table 2, fig. 1a and b).

Similar results were obtained when TSS was considered. After 1 and 2 h of O₃ exposure TSS significantly increased when compared with air exposure in group A subjects, but not in group B subjects (table 2). When compared with pre-exposure evaluation, TSS increased after 1 and 2-h O₃ exposure in group A subjects, and only at 2 h in group B subjects. No changes were observed in TSS after air exposure in either of the two groups examined.

There was no significant difference in maximum FEV₁ fall following HS inhalation between air and O₃ exposure (table 2).

Cell percentages and soluble markers in induced sputum

Median values of sputum inflammatory cell percentages after air exposure were similar in the two groups (table 3).

The percentage of sputum neutrophils was significantly higher 6 h after O₃ exposure compared with air exposure in all subjects, in those with mild intermittent asthma (group A) and in those with mild persistent asthma (group B) (table 3), and the increase was consistent in each subject (fig. 2). The sputum eosinophil percentage, which was already high after air exposure, decreased after O₃ exposure in both groups of subjects, probably owing to the increase in neutrophil percentages; the difference was, however, not significant (table 3).

The median value of total inflammatory cells in the induced sputum was not different between air and O₃ exposure in both groups examined (group A: $4.92 \pm 2.71 \times 10^6$ versus $3.51 \pm 4.43 \times 10^6$; group B: $2.47 \pm 1.93 \times 10^6$ versus $3.50 \pm 1.93 \times 10^6$, after air and O₃ respectively) nor the percentage of noninflammatory squamous cells (group A: 24% (4.6–67.6) versus 35% (4.8–74); group B: 33% (13–72) versus 26% (2.4–71) after air and O₃ respectively).

There was no significant change in ECP levels in the sputum supernatant obtained after either air or O₃ exposure in both groups ($298.8 \mu\text{g}\cdot\text{L}^{-1}$ (103–1887) versus $238 \mu\text{g}\cdot\text{L}^{-1}$ (75.4–4250) for group A; $160.1 \mu\text{g}\cdot\text{L}^{-1}$ (123.32–449.36) versus $324.2 \mu\text{g}\cdot\text{L}^{-1}$ (129.1–917.4) for group B).

IL-8 levels were significantly higher in sputum supernatant collected 6 h after the end of O₃ exposure than after air exposure in mild intermittent asthmatic subjects, but not in mild persistent asthmatic subjects (fig. 3). IL-8 levels after air exposure were not significantly different between two groups.

Discussion

These results show that exposure to 0.26 ppm of ozone for 2 h elicits in untreated mild intermittent asthmatic

Fig. 1. — Individual values of forced expiratory volume in one second (FEV₁) measured before (Pre), 1 and 2 h after the beginning of ozone (a and c) and air exposure (b and d) in mild intermittent asthmatic subjects (a and b) and in mild persistent asthmatic subjects (c and d).

Table 3. – Median values (range) of sputum cell percentages after air and ozone exposure in the two groups examined

	Group A Mild intermittent		Group B Mild persistent	
	Ozone	Air	Ozone	Air
Macrophages	23.5 (2.4–61.2)	43.3 (17–75.3)	31.9 (15.1–69)	57.3 (16.8–81.8)
Lymphocytes	0.8 (0–1.8)	0 (0–4.3)	0.3 (0–1)	9 (0–1.5)
Neutrophils	70.2 (28–87)	26.6 (8.6–73.2)*	62.1 (25–82.4)	27.9 (14.4–39.6)*
Eosinophils	4.3 (0–31.6)	14 (3–36.8)	2.8 (2–34.9)	10 (3.1–56.1)

*: $p < 0.05$, between post-air and post-ozone exposure.

subjects a greater airways response than in treated mild persistent asthmatic subjects, both in terms of clinical and functional response, and in terms of biochemical and cellular changes in induced sputum.

The pro-inflammatory activity of O_3 on human airways is well known. Up until now most human studies used BAL as a research tool to investigate the airway inflammatory response to O_3 exposure. Regarding healthy subjects, a significant increase in percentage of neutrophils in BAL fluid has been found 6–18 h after O_3 exposure [7, 18]. The strongest evidence of O_3 -induced airway injury in normal subjects has been given by ARIS *et al.* [19] who demonstrated by histological data an increase in neutrophils in O_3 -exposed bronchial tissue compared with that exposed to air. More recently, both BASHA *et al.* [9] and SCANNELL *et al.* [10] also used BAL in asthmatic subjects to study inflammatory response to O_3 exposure. They demonstrated that O_3 elicits a greater inflammatory response in asthmatics than in normal subjects.

Only few studies have used the analysis of the induced sputum to investigate the airways inflammatory response to outdoor pollutants [11, 20, 21]. FAHY *et al.* [21] showed that exposure of healthy subjects to 0.4 ppm O_3 for 2 h was associated with significantly greater increases in sputum neutrophil percentage and in myeloperoxidase (MPO) concentration than air exposure. HILTERMANN *et al.* [11] demonstrated that exposure to 0.4 ppm O_3 for 2 h induced a significant increase in sputum neutrophils in normal and in mild asymptomatic asthmatic subjects. The present data confirm that the pre-existing eosinophilic inflammation was not increased by ozone exposure in asthmatic subjects, as shown by the lack of increase in eosinophil percentage

and ECP levels in the induced sputum obtained after O_3 exposure.

Most previous studies have examined the effect of O_3 on mild asymptomatic asthmatic subjects not requiring regular treatment. In this category of patient, the results of the present study are in agreement with those obtained by other authors. Different data was obtained in mild persistent asthmatic subjects who required regular treatment with inhaled corticosteroids and long-acting β_2 -agonists for the relief of symptoms. The presence of daily asthma symptoms was confirmed in this group by PEF and symptom monitoring conducted preliminarily during a short-term withdrawal of regular treatment. In this group of patients, a significant increase in sputum neutrophils was not accompanied by a significant increase in sputum IL-8 concentration and was associated with a mild change in FVC, FEV1 and TSS in comparison with untreated mild intermittent asthmatic subjects. To the best of the authors' knowledge, no previous study has compared the effect of O_3 in groups of subjects with different asthma severity.

The mild effect of O_3 on regularly treated asthmatics cannot be ascribed to a direct effect of antiasthma drugs on acute airway response to O_3 , because treatment was withdrawn 72 h before O_3 exposure. It cannot be excluded that a longer withdrawal of antiasthma drugs before O_3 exposure could have determined different results. This wash-out time period was chosen for two reasons: 1) to avoid any direct effect of antiasthma drugs on the airway response to ozone; and 2) subjects were not able to withdraw asthma treatment for a longer period of time. Previous

Fig. 2. – Individual values of sputum neutrophil percentages after air and after ozone in a) mild intermittent asthmatic subjects (group A) and b) mild persistent asthmatic subjects (group B).

Fig. 3. – Median values, interquartiles, 90th percentiles and range of interleukin (IL)-8 concentrations in the sputum supernatant after air and ozone in a) mild intermittent asthmatic subjects (group A) and b) mild persistent asthmatic subjects (group B).

studies have used similar wash-out periods [22]. By contrast, it is possible that long-term treatment with inhaled corticosteroids could have changed the pattern of the airway response to O₃, and this different reactivity could persist some days after the withdrawal of treatment. An effect of budesonide on O₃-induced airway inflammation has been demonstrated in dogs [23]. Inhaled corticosteroids have been reported to increase the level of neutral endopeptidase (NEP) in bronchial mucosa, to improve vascular permeability and to modulate the expression of adhesion molecules on epithelial and endothelial cells [24]. All these mechanisms are presumed to be involved in the inflammatory response of the airways to inhaled oxidants such as O₃ [25]. This hypothesis should be investigated by a different study protocol.

Few studies have reported the influence of antiasthma drugs, such as systemic steroids on the airway response to O₃ [26, 27], showing no significant effect. Only indomethacin has been demonstrated to be able to prevent a functional airway response to O₃ in humans [28]. However, all these studies concerned normal subjects and a short course of treatment; therefore, the lack of pre-existing airway inflammation and the short duration of treatment could have determined the failure in preventing O₃-induced airway inflammation by antiasthma drugs.

A further possible explanation of the milder airway response to O₃ in subjects with mild persistent asthma could be a greater activity of antioxidant properties in the airway mucosa in subjects with more severe airway inflammation. This hypothesis has not been confirmed, and some previous studies did not show any difference in the antioxidant levels in bronchial or nasal lavage of subjects with different airway response to inhaled oxidants [29, 30].

A further explanation of the difference in the increase of symptom score after O₃ between the two groups could be due to a blunted perception of dyspnoea and other irritant symptoms of the upper airways in subjects with mild persistent asthma in comparison with patients with mild intermittent asthma [31].

The discrepancy between the increase in sputum neutrophils and the lack of increase in sputum IL-8 in mild persistent asthmatics suggests that chemotactic factors other than IL-8 could be involved in the recruitment of neutrophils in the airways after O₃ exposure. *In vitro* studies have shown the release of several chemotactic factors, including leukotriene B₄, from epithelial cells exposed to O₃ [32].

The sample size of the examined subjects was low in this study, but not different from that reported in several previous studies [4, 6, 11, 21]. There was also a small nonsignificant difference in the mean age of the two groups. A lower airway response in older subjects has been reported, but it was evident in healthy adults >50 yrs of age [33], and the relationship between FEV₁ changes and age was weak [34].

The asthmatic subjects were not pretreated with inhaled β_2 -agonist before HS inhalation to assess the airway responsiveness to hypertonic challenge. At the time of the study, all subjects had an FEV₁ in the normal range, and reported occasional asthma symptoms. In these subjects, maximum fall in FEV₁ after HS inhalation was well tolerated. However, as recommended by several authors, β_2 -agonist pretreatment is mandatory in stable asthmatics,

as well as in the presence of airway obstruction or severe bronchial hyperresponsiveness [35].

In conclusion, it was demonstrated that the degree of airway response to a short-term exposure to ozone is different in asthmatic subjects, depending on the severity of the disease and the requirement of regular anti-inflammatory treatment. Both the functional response and the increase in interleukin-8 sputum concentration were reduced in subjects with mild persistent asthma in comparison with subjects with mild intermittent asthma. The available data do not allow us to define whether this difference depends on the severity of the disease or on the regular anti-inflammatory treatment.

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