Serum levels of eosinophilic cationic protein (ECP), myeloperoxidase (MPO), Lipid peroxidation products, interleukin (IL)-5 and interferon (IFN) – γ in children with bronchial asthma at acute asthma attack and remission*

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SUMMARY: Kalaycı Ö, Saraçlar Y, Kılınç K, Şekerel BE. Serum levels of eisonophilic cationic protein (ECP), myeloperoxidase (MPO), lipid peroxidation products, interleukin (IL)-5 and interferon (IFN)-γ in children with bronchial asthma at acute asthma attack and remission. Turk J Pediatr 2000; 42: 9-16.

We determined serum levels of myeloperoxidase (MPO), eosinophilic cationic protein (ECP), reactive oxygen species measured as thiobarbituric acid reactive substances (TBARS), interleukin (IL)-5, and interferon (IFN)-γ in 14 asthmatic children during an asthma attack and remission. Twelve healthy children served as controls. In atopic asthmatics, asthma attack resulted insignificant elevations of ECP, MPO, and TBARS compared to remission. TBARS levels were also higher at remission compared to controls. However, there was a great deal of overlap in the values of asthmatics and controls. IL-5 and IFN-γ were detectable at low levels and only in a few patients. These results provide further evidence for participation of eosinophils, neutrophils and reactive oxygen species in the pathogenesis of acute asthma, and suggest that their products may be used in monitoring asthma attack. Serum IL-5 and IFN-γ levels are not appropriate for use in the follow-up of asthmatic children.

Key words: eosinophilic protein, interleukin (IL)-5, interferon (IFN)-γ, lipid peroxide, myeloperoxidase.

Recent studies have shown that airway inflammation is a persistent feature of asthma even in its mildest forms and during asymptomatic periods[1]. A subgroup of T lymphocytes (Th-2) that synthesize mainly interleukin (IL)-4 and IL-5 and little interferon (IFN)-γ and eosinophils have a key role in the inflammation of allergic asthma[2-4]. Although the role of neutrophils is not as clear, there is some data to indicate that neutrophils are important in the late asthmatic response[5]. Production of reactive oxygen species (ROS), a major mechanism by which both neutrophils and eosinophils participate in human defense, has also been implicated in the pathogenesis of bronchial asthma[6].

Although inflammation is the underlying factor, markers of inflammation to aid in diagnosis and follow-up of asthma have not entered into clinical use; currently, clinical findings and pulmonary function tests are the only tools that serve this purpose. This presents some difficulties, especially for childhood asthma, as symptoms of the disease can be quite nonspecific and pulmonary function tests are not reliable in children younger than five years of age. Furthermore, there can be a great deal of discrepancy between pulmonary function tests and symptoms, even in adults[7]. Clearly, easily measurable objective markers to aid in the diagnosis and follow-up of asthma would be quite useful. Among these markers are serum levels of eosinophilic cationic protein (ECP), IL-5[8], myeloperoxidase (MPO)[9], and lipid peroxidation products (as an indirect measure of ROS)[10,11]. ECP, as a marker of eosinophil

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activity, has been the subject of intense investigation, and it is generally believed to be a good marker of disease activity[8,12,13]. Unlike with eosinophils, the data about neutrophils is scarce and controversial[14-17]. MPO is believed to reflect neutrophil activity, as 95 percent of the protein is derived from these cells, whereas the remaining five percent is contributed by monocytes. Therefore, MPO is believed to be an appropriate marker for neutrophil activation.

In vitro studies have clearly defined the significance of ROS in bronchial asthma[6,18-20]. However, since ROS are very quickly removed or scavenged by local mechanisms, it is very difficult to measure them directly in biologic systems. ROS can act on membrane phospolipids and cause lipid peroxidation. Measurement of thiobarbituric acid reactive substances (TBARS) is a good marker for ROS production[21], There are conflicting reports about the serum levels of lipid peroxides and their significance in bronchial asthma[10,11,22].

The aim of this study was to measure the serum levels of ECP, MPO, TBARS, IL-5, and IFN-γ in a group of asthmatic children. Levels were determined during a period of hypoxia (acute asthma attack) and 30 days after discontinuation of all medical treatment (remission). Patients with possible confounding factors like medication usage or presence of infections were excluded from the study. We also investigated whether these indices had any relation to the level of hypoxia and pulmonary function parameters.

Material and Methods

Patients

Fourteen children, aged 3-15 years, who presented with an acute asthma attack to the emergency department of Hacettepe University, İhsan Doğramacı Children's Hospital were included in this study. The patients had to fulfill the following criteria in order to be eligible for the study:

1. Moderate to severe asthma attack[23].
2. Hypoxia: oxygen saturation < 95 percent.
3. Improvement (15%) in FEV1 after salbutamol inhalation during the asthma attack or following recovery.
4. No bronchodilator or antiinflammatory treatment before or during presentation to the hospital and no history of immunotherapy.
5. No current infection or systemic disease except bronchial asthma.

Control Group

Twelve healthy age-matched children without any systemic or atopic diseases served as the control group. In order to ensure the control group had no atopy, their sera were tested for the presence of specific IgE for a combination of antigens (All Screen Inhalation, Melja Diagnostik, Kassel, Germany): Dermatophagoides pteronyssinus, cat, dog, phleum pratense, secale cereale, cladosporium, artemisia vulgaris, and betula verrucosa. To be included in the study the children had to have a negative result on this test. The sera of the control group, like that of the study population, were tested for ECP, MPO, IL-5, IFN-γ and TBARS. Only eight samples could be tested for ECP, where as all 12 samples were tested for the other parameters.

Design

Blood (15-20 ml) was drawn from the radial artery of each patient upon presentation with an acute asthma attack. From this sample, blood-gas determination, complete blood count and differential, and total eosinophil count[24] were done, and serum was separated and stored at -20 °C until analysis for measurement of total and specific IgE, ECP, MPO, IL-5, IFN-γ and TBARS. Pulmonary functions were also measured (2130 PFT system, Sensor Medics, CA, USA). All patients were then treated according to a standard protocol involving nebulized salbutamol and oral prednisolone. Patients were followed in the emergency room until their oxygen saturation was > 95 percent at room air or their FEV1 was > 75 percent of the predicted. After discharge from the emergency room, oral methylprednisolone was tapered to zero in 7-10 days. During this period all patients used inhaled salbutamol via a spacer starting with 200 μg every two hours and then at longer intervals. Upon recovery, both corticosteroid and salbutamol were discontinued and patients were called for a follow-up visit in 30 days, when pulmonary functions and blood tests were repeated. One day later, all patients were skin tested with common aeroallergens and food allergens.

Assessment of Atopy

Patients were skin-prick tested with a battery of antigens including house dust mite species,

animal antigens, American cockroach, grass weed and tree pollens, milk and egg white. An induration 3 mm greater than the negative control associated with surrounding erythema was considered positive[25]. If the results of skin-prick testing were negative, intradermal tests were done with the same antigens. Each patient's serum was then tested for the presence of specific IgE with the relevant antigen(s) (AlaSTAT, Diagnostic Products Corporation, Los Angeles, CA, USA). To be considered atopic, patients had to have at least one positive skin prick and a greater than class 2 specific IgE with the antigen(s). Nonatopic patients had negative skin-prick and intradermal tests and a less than class 1 specific IgE to aeroallergens.

Blood Collection and Handling

Blood samples were drawn from the radial artery in the study group and from the antecubital vein in the control group and were transferred to a serum separating tube (Becton Dickinson, Mountain View, CA, USA) and allowed to clot at room temperature for 60 minutes (min). Samples were then centrifuged at 1350 g for 10 min. The serum was separated, aliquoted and stored at -20 °C until analysis.

Eosinophilic cationic protein (ECP) and MPO were measured by radioimunosorbent assay (RIA) using Pharmacia kits (Kabi Pharmacia Diagnostics AB, Uppsala, Sweden), and IL-5 and IFN-γ by enzyme linked immunosorbent assay (ELISA) using cytoscreen immunoassay (Biosource International, CA, USA). The lowest detection limit was < 4 pg/ml for both IL-5 and IFN-γ.

Serum lipid peroxide levels were determined measuring thiobarbituric acid (TBA) reactivity as described by Wade and van Rij[26]. Briefly, 200 µl of trichloroacetic acid (TCA) (25 g TCA in 10 ml distilled water) was added to 1 ml of serum. The mixture was centrifuged at 1000 g for 10 min, and the precipitate was reacted with 1 ml of 0.67% TBA (w/v). The samples were heated at 100 °C for 30 min. After centrifugation, the absorption of malondialdehyde (MDA)-TBA chromogen was measured at 532 nm. Tetramethoxy propane was used as malondialdehyde standard. TBA reactivity was calculated as micromole MDA per liter (µM).

Statistical Analysis

All statistical analyses were done using SPSS 6.0 statistical program. For comparisons between the acute attack and remission periods of asthma patients, Wilcoxon paired samples test was used, and Mann Whitney U test was used for comparison of asthma patients with controls. Correlations between parameters were studied using Pearson's test. Values are expressed as mean ± standard error of the mean (SEM). A significance level of five percent was used unless stated otherwise.

Results

Fourteen patients that presented with moderate to severe asthma attack to the emergency room of İhsan Doğramacı Hospital were included in the study. Of the 14 patients, 11 were atopic and three nonatopic. Nonatopic patients' asthma attacks were due to exposure to cigarette smoke in two and to automobile exhaust in one patient. Characteristics of the patients are summarized in Table I. Mean ages of the atopic patients and controls were 9.3 ± 1.2 and 10.1 ± 1.0 years, respectively ($p > 0.05$). The results of the three nonatopic patients are summarized in Table II and those of atopic asthmatics and controls are given below.

Table I. Characteristics of Asthma Patients

Patient no.	Age (years), gender	Specific IgE class*			Total IgE (IU/ml)
		Grass	Weed	Dust mite	
1	8,F	3			1988
2	5,M			4	533
3	9.5,F	3	4	4	1022
4	10,M		2		48
5	14,M	1		4	507
6	8,M	4	3	3	813
7	13,M	1		2	171
8	3,F			2	82
9	13,M	4	2		1450
10	15,M	4		3	2276
11	4,M	4			35
12#	12,M	–	–	–	13
13#	7,M	–	–	–	44
14#	5,M	–	–	–	63

*: Patients 1, 3, 4, 6 and 7 also had positive skin prick tests with trees and patient 11 with cat, but specific IgE results for these antigens were not available.

#: Nonatopic patients.

Table II. Results of the Nonatopic Asthmatic Children (Asthma Attack/Remission)

Patient no.	Leukocyte count (mm^3)	Eosinophil count (mm^3)	FEV1 (% predicted)	PaO_2 mmHg	ECP µg/L	MPO mg/L	TBARS µM
12	5200/5600	105/120	69/79	73/86	8/4	406/250	5.41/3.67
13	17500/7900	200/300	38/110	71/138	16/40	482/615	4.28/3.79
14	7500/7500	225/300	59/102	75/96	7/14	303/485	3.28/3.00

ELP: eosinophilic cationic protein; MPO: myeloperoxidase; TBARS: thiobarbituric acid reactive substances.

Leukocyte and Eosinophil Counts: The mean leukocyte count was 9,845 ± 1,161 and 7,154 ± 517/mm^3 at asthma at asthma attack and remission, respectively ($p > 0.05$). Similarly, the eosinophil count did not differ at asthma attack (336 ± 120 mm^3) and remission (290 ± 58 mm^3) ($p > 0.05$). Evaluation of the differential counts failed to show any difference in the numbers of neutrophils, lymphocytes, or monocytes between these two periods (data not shown).

FEV1 and PaO_2: Pulmonary functions were measured in eight patients. The mean of FEV1 as percentage predicted was 52.5 ± 5.2 at asthma attack and 94.3 ± 4.8 at remission (Fig. 1a); the difference was significant ($p = 0.01$). The mean of PaO_2 was also significantly lower at asthma attack (68.9 ± 1.8 mmHg) compared to remission (102.0 ± 4.0 mmHg) ($p = 0.003$) (Fig. 1b).

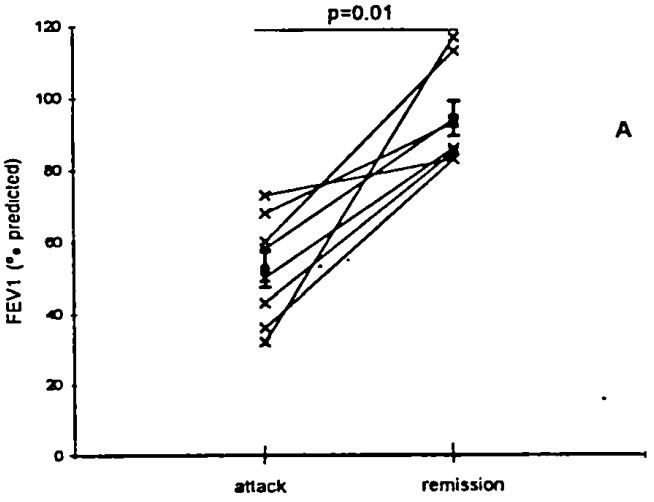

Fig. 1. FEV1 (A) and PaO_2.

ECP: The mean of ECP at asthma attack (50.1 ± 17.1 µg/L) was significantly higher than at remission (12.4 ± 2.1 µg/L) ($p = 0.003$). However, the mean ECP of controls (8 patients) (19.5 ± 2.8) was not statistically different from the value obtained at remisision (Fig. 2a).

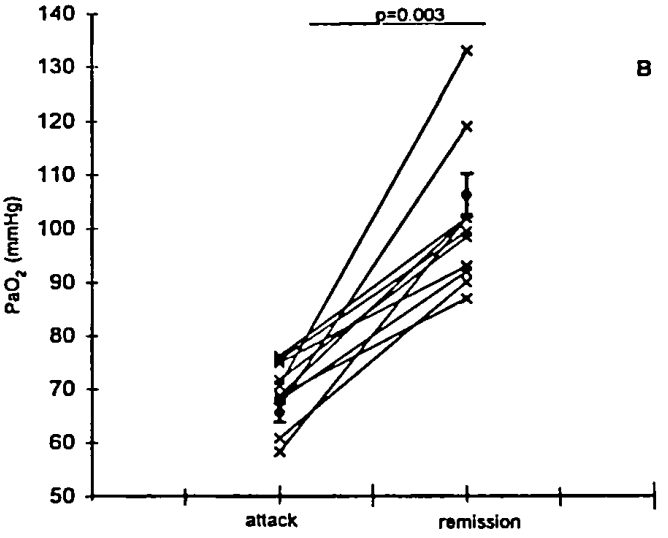

Fig. 1. FEV1 (B) and values and their means in individual atopic patients.

MPO: The results obtained for MPO were similar to those for ECP. Although a difference was observed between asthma attack (664.3 ± 118.4 µg/L) and remission (323.2 ± 40.4 µg/L) ($p = 0.004$), values of the control group (399.3 ± 48.6 µg/L) were not statistically different from the values obtained at remission (Fig. 2b).

TBARS: Atopic asthmatics had a significantly higher serum TBARS at asthma attack (4.1 ± 0.2 µM) compared to remission (2.8 ± 0.2 µM) ($p = 0.003$) (Fig. 2c). The mean of controls was 2.2 ± 0.2. The difference between the remission period and the controls just failed to reach statistical significance ($p = 0.06$). In fact, when atopic and nonatopic asthmatics were pooled, this difference actually reached statistical significance ($p = 0.018$) (Fig. 2d). However, there was a notable overlap in these values.

IL-5 and IFN-γ: Both cytokines were undetectable in the majority of samples. In the few samples where levels were within detection limits, their concentrations were quite low and close to the lower limit of detection.

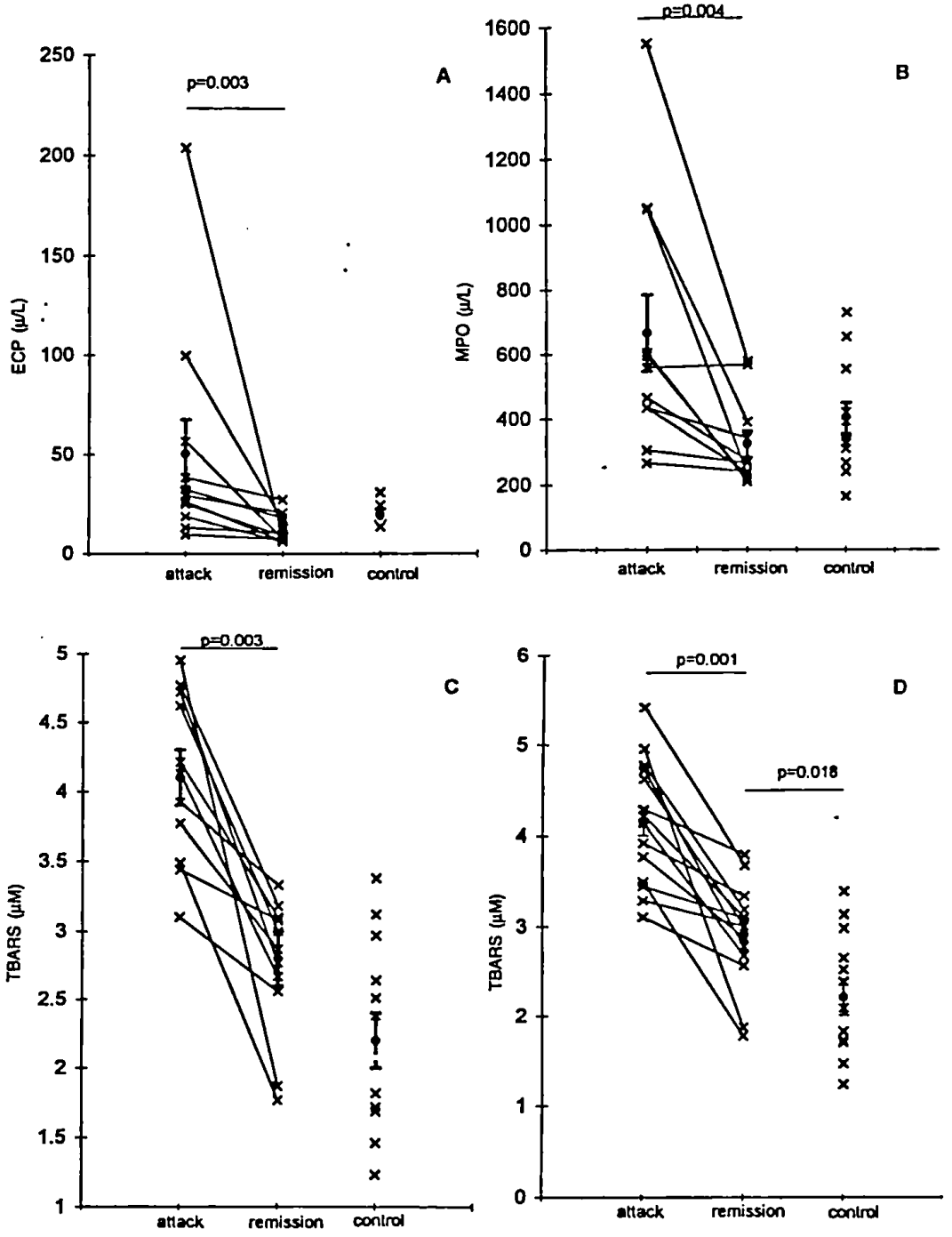

Fig. 2. ECP (A), MPO (B), and TBARS (C) values and their means in atopic patients at asthma attack and remission. 2D depicts the TBARS levels of all asthmatic children, including both atopic and nonatopic patients.

Correlation Analyses

As expected, FEV1 and PaO_2 showed a very high correlation in atopic patients ($r = 0.88$, $p < 0.001$). Similarly, ECP and eosinophil counts also showed a significant correlation in this group ($r = 0.69$, $p < 0.001$). However, ECP failed to show a significant correlation with either FEV1 and PaO_2 ($p > 0.05$ for both). MPO, on the other hand, while showing a significant correlation with PaO_2 ($r = -0.57$, $p = 0.006$) (Fig. 3a), did not disclose any correlations with other parameters studied, including leukocyte count.

Thiobarbituric acid reactive substances (TBARS) showed a high and significant correlation both with PaO_2 ($r = -0.56$, $p = 0.002$) (Fig. 3b), and with FEV1 ($r = -0.57$, $p = 0.0069$ (Fig. 3c).

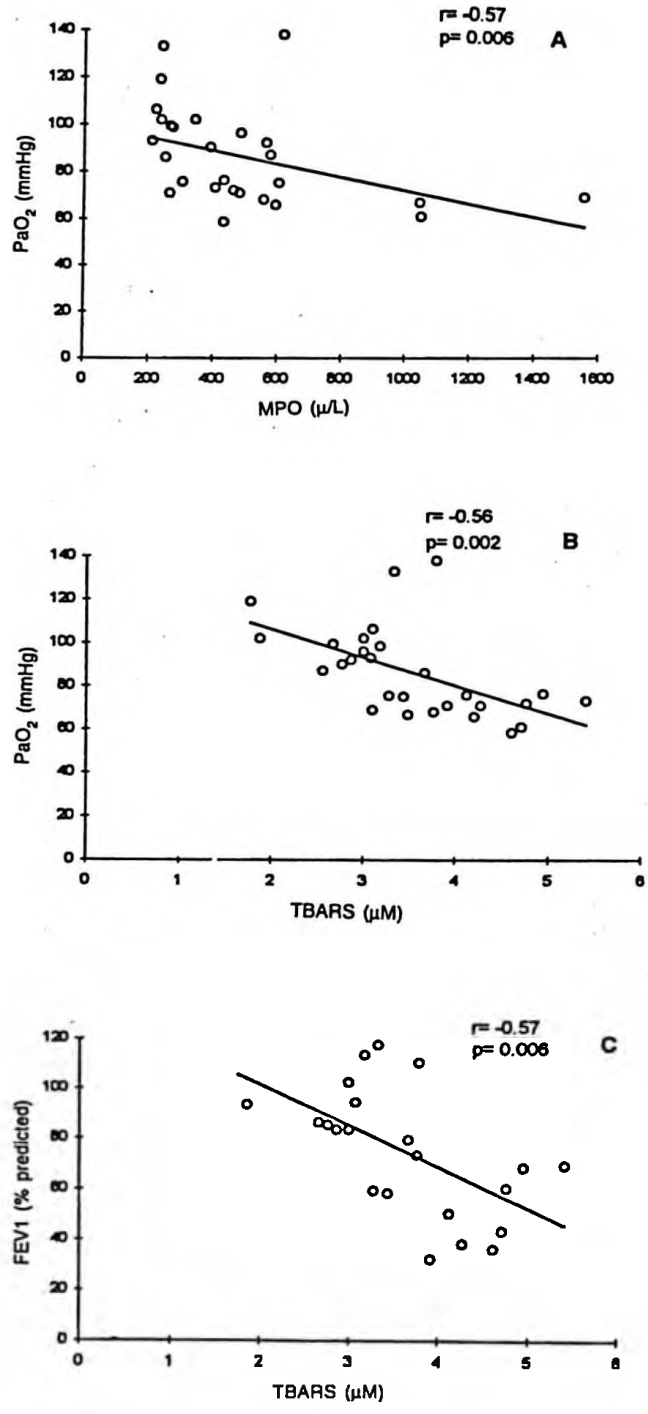

Fig. 3. Correlations between MPO-PaO_2 in atopic asthmatics (A), and TBARS-PaO_2 (B) and TBARS-FEV1 (C) in all asthmatic children, including both atopic and nonatopic patients.

Discussion

The results of our study confirm the participation of eosinophils, neutrophils, and reactive oxygen species in acute asthma, and further suggest that their products may be used as objective markers in the follow-up of acute asthma attack. However, the significant overlap observed in controls and asthmatics in all three variables indicates that they are not useful to differentiate asthmatics from normal controls. Furthermore, due to the design of our study, we cannot draw conclusions regarding the utility of these markers in the long term follow-up of chronic asthma. The other markers investigated in this study, measurement of serum IL-5 and IFN-γ levels, failed to produce any consistent results. In fact, they were undetectable in most of the samples. However, it should be pointed out that our findings do not contradict the importance of local production of

these cytokines. It is well documented that eosinophils are key effector cells in atopic asthma. Although total eosinophil count has been suggested to indicate the ongoing inflammation in asthma, it is claimed that eosinophilic proteins better reflect eosinophilic activity[13,27,28]. Our findings are also in favor of this hypothesis. It is difficult to reach conclusions regarding ECP in nonatopic patients because of their limited number. However, in two nonatopic patients, serum ECP level was actually higher in remission that it was in acute attack, a trend that was not observed in any of the atopic patients This finding implies that serum ECP may not provide valuable information in nonatopic patients. The reports about ECP in the medical literature are conflicting[29-32]. As suggested by Motojima et al.[8], these results may stem from the differences in the patients, in the severity of asthma, in medication usage and in serum separation techniques.

Myeloperoxidase (MPO) levels obtained at the time of an asthma attack, at remission and in controls followed a similar trend to those of ECP. In addition, MPO correlated significantly with PaO_2, suggesting that hypoxia contributed significantly to the increases in MPO and supporting our previous observations in rats[33]. Similar to ECP, MPO levels also decreased in 10 of 11 atopic patients after the asthma attack. In one patient only the level remained stable. This consistent trend was not observed in nonatopic asthmatics. In two nonatopic patients the levels of MPO at acute asthma attack were actually lower than they were at remission. Taken together, these findings suggest that a combination of the atopic state and hypoxia may have contributed to the activation of leukocytes resulting in increased MPO. This study, like many others, supports the notion that neutrophils play a significant role in asthma. In the study by Scher et al.[9], for instance, MPO was found to increase in asthmatic patients. Similar to our study, this increase did not correlate consistently with increases observed in eosinophilic proteins, nor with pulmonary function tests.

Thiobarbituric acid reactive substances (TBARS) differend from ECP and MPO in that asthmatics had significantly higher values even at remission compared to controls. Still, it is difficult to claim that this finding reflects an ongoing oxidant stress even during asymptomatic periods in asthma because there was a remarkable overlap in the values obtained in asthmatics and controls. Most of the studies that have investigated the role of ROS in asthma are conducted in in vitro and ex vivo systems[34-36]. The studies that have looked into this question by determining lipid peroxidation products are limited and have produced conflicting results[10,11,22]. Owen et al.[10] showed that a lipid peroxidation product, phospholipid esterified 9, 11 linoleic acid (PL-9, 11-LA), is increased in serum in asthmatic patients compared to controls. They have further found that this increase is more pronounced in acute compared to chronic asthma. These findings are in accordance with our study. In another study, however, totally different results were obtained[22]. In this study PL-9, 11-LA was serially measured in eight patients and failed to show any increase at any time point starting from an acute asthma attack. Five of these patients were using antiinflammatory treatment while the remaining three were on bronchodilators. Many drugs, including teophyllin, are known to effect lipid peroxide levels[37,38]. The results of this study may have been confounded by the concomitant medication usage. Another major difference between our and the two above referenced studies[10,22] is that both of the latter were conducted in adult populations which may, in part, be responsible for the different results.

Further studies using larger sample sizes and also serial measurements are needed to define the precise value of these parameters in the follow-up of asthma.

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