

High mobility group box 1: Biomarker of inhaled corticosteroid treatment response in children with moderate-severe asthma

Sara Manti, M.D.,¹ Salvatore Leonardi, M.D.,² Giuseppe F. Parisi, M.D.,² Dominique De Vivo, M.D.,¹ Annamaria Salpietro, M.D.,¹ Antonietta Spinuzza, M.D.,¹ Teresa Arrigo, M.D.,¹ Carmelo Salpietro, M.D.,¹ and Caterina Cuppari, M.D.¹

ABSTRACT

Background: High mobility group box 1 (HMGB1) is abnormally expressed in serum and sputum of patients with allergic asthma.

Objective: The aim of this study was to investigate the role of HMGB1 as guidance for treatment management of children with asthma.

Methods: Thirty children with asthma and 44 healthy children were enrolled. The patients were classified according to Global Initiative for Asthma Guideline disease severity criteria. Sputum HMGB1 levels and lung function index (percentage forced expiratory volume in 1 second [FEV₁%]) were recorded in the cohort study at baseline (T0) and after 3 (T3) and 6 (T6) months of inhaled corticosteroids (ICS) treatment.

Results: Sputum HMGB1 levels were significantly higher in all the patients with asthma ($p < 0.001$). An inverse correlation between sputum HMGB1 levels and pulmonary function parameters was observed only in the children with moderate asthma (T0: FEV₁%, $r = -0.9891$, $p < 0.001$; T3: FEV₁%, $r = -0.6763$, $p < 0.001$; T6: FEV₁%, $r = -0.5419$, $p < 0.05$) and in the children with severe asthma (T0: FEV₁%, $r = -0.8696$, $p < 0.001$; T3: FEV₁%, $r = -0.6477$, $p < 0.05$; T6: FEV₁%, $r = -0.8627$, $p < 0.001$). After ICS treatment, a significant decrease of sputum HMGB1 levels was noted in moderate (T0 [93.44 ± 20.65 ng/mL] versus T3 [77.96 ± 1.81 ng/mL] versus T6 [67.75 ± 3.01 ng/mL]; $p < 0.0001$) and in the children with severe asthma (T0 [130.3 ± 7.48 ng/mL] versus T3 [156.9 ± 1.09 ng/mL] versus T6 [116.08 ± 4.77 ng/mL]; $p < 0.0001$) data are mean ± standard deviation, respectively. The area under the receiver operating characteristic curve, performed to define the diagnostic profile of sputum HMGB1 levels in identifying the children with asthma, was 0.713.

Conclusion: In addition to the findings that HMGB1 is a sensitive biomarker of allergic asthma in children, our data demonstrated a significant correlation between the decrease of HMGB1 levels and a successful treatment response.

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Asthma is a chronic inflammatory airway disease characterized by recurrent bronchospasm, bronchial hyperresponsiveness, and reversible airway obstruction.^{1–3} In accordance to the Global Initiative for Asthma Guideline (GINA), asthma is classified as mild, moderate, or severe disease.¹ However, it is well known that clinical characteristics (symptoms and exacerbation), limitation of activities, and reduction in lung function tests are not very sensitive. Consequently, treatment and clinical decision-making are burdened by remarkable heterogeneity. To date, although inhaled corticosteroids (ICS) are greatly recog-

nized as the first-choice maintenance treatment in children with moderate-severe allergic asthma, the exact treatment duration is still a matter of debate.¹

To prevent under- and overtreatment of children with allergic asthma and to better personalize the disease management, new noninvasive biomarkers of airways inflammation are being developed.^{4,5} In this regard and in accordance to previous studies,^{6–10} we recently provided evidence that sputum high mobility group box-1 (HMGB1), an alarmin that promotes an immediate immune response to tissue damage, is a sensitive biomarker in children with stable off-therapy allergic asthma, and it also seems to have an inverse correlation with lung function,¹¹ which indicates its use as a biomarker of clinical response to treatment in children.^{12–14} To date, data with regard to the association between sputum HMGB1 levels and asthma control status have not been reported. Therefore, the aim of this study was to investigate the role of HMGB1 as a novel inflammatory biomarker that could be used as guidance for management of treatment for children with asthma.

From the ¹Unit of Pediatric Genetics and Immunology, Department of Pediatrics, University of Messina, Messina, Italy, and ²Department of Clinical and Experimental Medicine, University of Catania, Catania, Italy

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Address correspondence to Salvatore Leonardi, M.D., Department of Clinical and Experimental Medicine, University of Catania, Via S.Sofia n.78, Catania, Italy 95123
E-mail address: leonardi@unict.it

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METHODS

Subjects and Experimental Design

A total of 74 white children were enrolled in the study. In particular, 30 children or adolescent subjects with asthma (18 boys and 12 girls, mean age [standard deviation {SD}], 10.56 ± 0.32 years) who had been referred to the Section of Immuno-Allergology, Genetics and Pediatric Immunology Unit, Department of Pediatrics, University of Messina, between September 2013 and December 2013, were enrolled in the asthma group. The patients were classified according to GINA disease severity criteria (mild, moderate, and severe).¹ Lung function test for the percentage forced expiratory volume in 1 second (FEV₁%) was performed.¹

Exclusion criteria were associated diseases (hepatic, infectious, or endocrine diseases; genetic syndromes; immunodeficiencies; neurologic and psychiatric problems), pulmonary or cardiac congenital malformations, chronic pulmonary disease (bronchopulmonary dysplasia, cystic fibrosis, bronchiolitis obliterans postviral infection), prematurity, small for gestational age, concurrent stridor, foreign body aspiration, contact with tuberculosis, and laryngomalacia. None of the patients had respiratory infections for at least 3 months before the beginning of the study. Before the start of the clinical study, only inhaled β_2 -agonists were administered to all the patients. The children had received ICS treatment according to GINA criteria.¹ In particular, moderate (>200 – $400 \mu\text{g}$) and high ($>400 \mu\text{g}$) daily doses of beclomethasone dipropionate were administered to the children with moderate allergic asthma and the children with severe allergic asthma, respectively, for 6 months. If necessary, inhaled β_2 -agonists were administered. Neither cromones nor other treatments capable of interfering with the results in the previous month the inclusion into the study, were allowed during the time of the study. Patients with poor medication adherence were also excluded.

Forty-four healthy children (22 boys and 22 girls, mean [SD], age 11.07 ± 2.2 years) were enrolled as healthy controls (the control group). Apart from asthma, inclusion and exclusion criteria were identical to those used for the asthma group. A complete physical examination of each child was performed by a physician at baseline (T0), 3 months (T3), and 6 months (T6). Demographic data and other clinical characteristics of the study subjects were collected by using questionnaires. Each child's height and weight were also measured. The institutional review board of the University of Messina approved the study, and written informed consent was obtained from the parents, and informed assent was obtained from the children and adolescents.

Skin-Prick Test and Immunoglobulin E Measurement

The skin-prick test and immunoglobulin E (IgE) measurement were performed at baseline. Particularly, the skin-prick test was performed by using a panel of aeroallergens, including, house-dust mite, mixed grass, *Parietaria*, birch, olive tree, *Alternaria*, epithelium cat, and epithelium dog (Allergopharma, Reinbek, Germany). Normal saline solution and histamine were used as negative and positive controls, respectively. Skin-wheal diameter was recorded at 15 minutes as the mean of two perpendicular measurements. A positive response was defined as a skin-wheal diameter of ≥ 3 mm compared with the negative control.¹ Total serum IgE levels were also determined in all the subjects, by using the ImmunoCAP100 system (Phadia, Uppsala, Sweden).

HMGB1 Measurement

Sputum HMGB1 levels were carried out at T0, at T3, and T6 after ICS therapy. HMGB1 values were measured by performing an enzyme-linked immunosorbent assay by using an HMGB1 Enzyme-linked Immunosorbent Assay Kit II (IBL, Shino-Test Corporation, Kanagawa, Japan). The limit detection was $0.2 \text{ ng/mL} + 2.6 \text{ SD}$ for a high sensitive range; the limit of quantification was 0.1 ng/mL , with coefficients of variation $\leq 20\%$ for a high sensitive range. The samples were diluted 1:5 in sample diluent formulation and added to microtiter plates, which were then incubated for 24 hours at 37°C . After washing, $100 \mu\text{L}$ /well of antihuman HMGB1 peroxidase conjugated monoclonal antibody was added, and the plates were incubated at room temperature for 2 hours. After washing $3,3',5,5'$ -tetramethylbenzidine dihydrochloride was added to each well. The enzyme reaction was allowed to proceed for 30 minutes at room temperature. The chromogenic substrate reaction was stopped by addition of stop solution (0.35 mol/L of sulfuric acid), and the absorbance of each well was determined on a microplate reader (enzyme immunoassay), at 450 nm . The HMGB1 concentrations were deduced from a standard curve, with a lower limit of detection of 0.2 ng/mL .

Sputum Induction

Sputum induction was performed at T0, T3, and T6. For sputum induction and processing, we used the guidelines suggested by the Task Force on Induced Sputum of the European Respiratory Society.¹⁵ All the subjects were instructed to wash their mouth thoroughly with water. Sputum induction was performed with a hypertonic saline solution (4.5%). The subjects were encouraged to cough deeply at 3-minute inter-

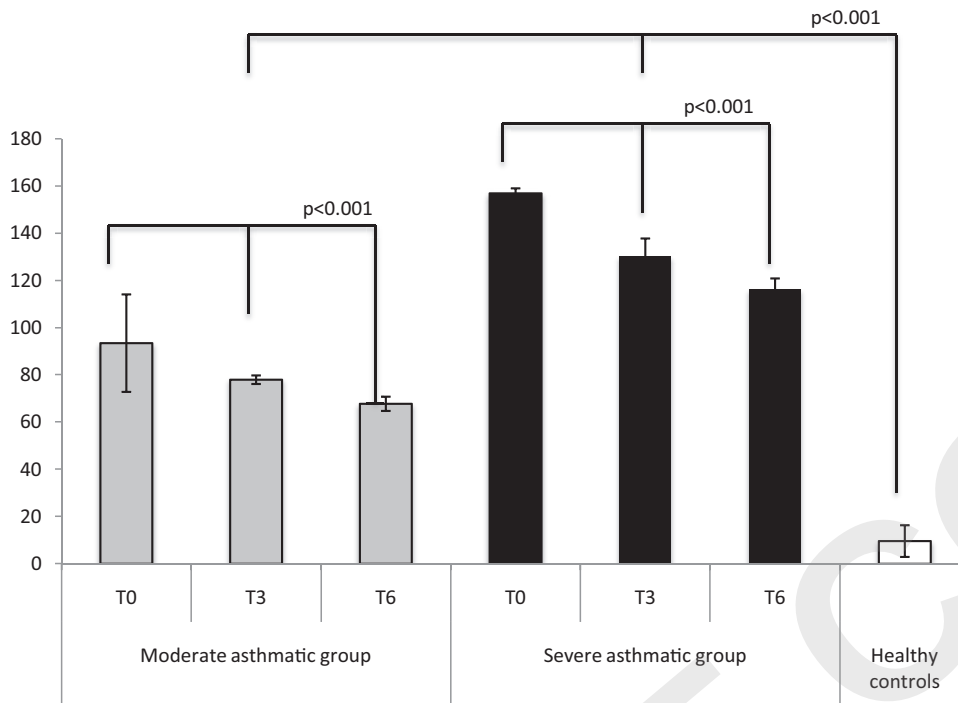


Figure 1. Sputum high mobility group box 1 (HMGB1) levels in asthma group and healthy controls.

Table 1 Clinical findings of children with asthma and healthy controls

	Children with Asthma (n = 30)	Healthy Controls (n = 44)	p
Age, mean $\pm$ SD, y	10.56 $\pm$ 0.32	11.07 $\pm$ 2.12	0.21
Boys/girls, no.	18/12	22/22	0.12
BMI, mean $\pm$ SD, kg/m ²	17.38 $\pm$ 0.23	17.49	0.11
Family history of asthma and/or atopy, no.	11	18	0.80
Serum total IgE, mean $\pm$ SD, IU/mL	162.67 $\pm$ 20.85	16.79 $\pm$ 7.31	<0.0001
Sputum HMGB1 levels, mean $\pm$ SD, ng/mL	125.02 $\pm$ 21.53	9.23 $\pm$ 3.71	<0.0001
FEV ₁ , mean $\pm$ SD, % predicted	66.35 $\pm$ 4.24	91.95 $\pm$ 3.27	<0.0001

SD = Standard deviation; BMI = body mass index; IgE = immunoglobulin E; HMGB1 = high mobility group box 1; FEV₁ = forced expiratory volume in 1 second.

vals. The subjects were told to periodically spit saliva into one container and expel sputum into another.¹⁵

Lung Function Test

A lung function test was performed at T0, T3, T6. Pulmonary function was measured in the laboratory by using spirometry (Lungtest 1000; MES Ltd., Kraków, Poland). FEV₁% was measured as the criterion standard parameter for airflow obstruction assessment. The best spirometric measure of at least three attempts was recorded for analysis. The values were expressed as percentage of predicted values, based on the child's age, sex, weight, and height.

Data Analysis

The data collected were statistically analyzed by using the statistical computer software SPSS, version 15.0. A *p*

value of <0.05 was considered statistically significant. An analysis of variance test and relative *post hoc* versus control tests were performed. Means (SDs) were calculated for the variables, and the *t*-test was used for comparisons. For the attributes, the percentages were calculated first and then the χ^2 test was used for comparisons. Correlations between HMGB1 levels and FEV₁% were calculated by using the Spearman correlation coefficient. Receiver operating characteristic (ROC) analysis was used to calculate the area under the curve for HMGB1 levels to find the best cutoff values capable of identifying children with allergic asthma.

RESULTS

Forty-four healthy children, 20 children with moderate asthma, and 10 children with severe asthma were recruited. No children affected by mild asthma were en-

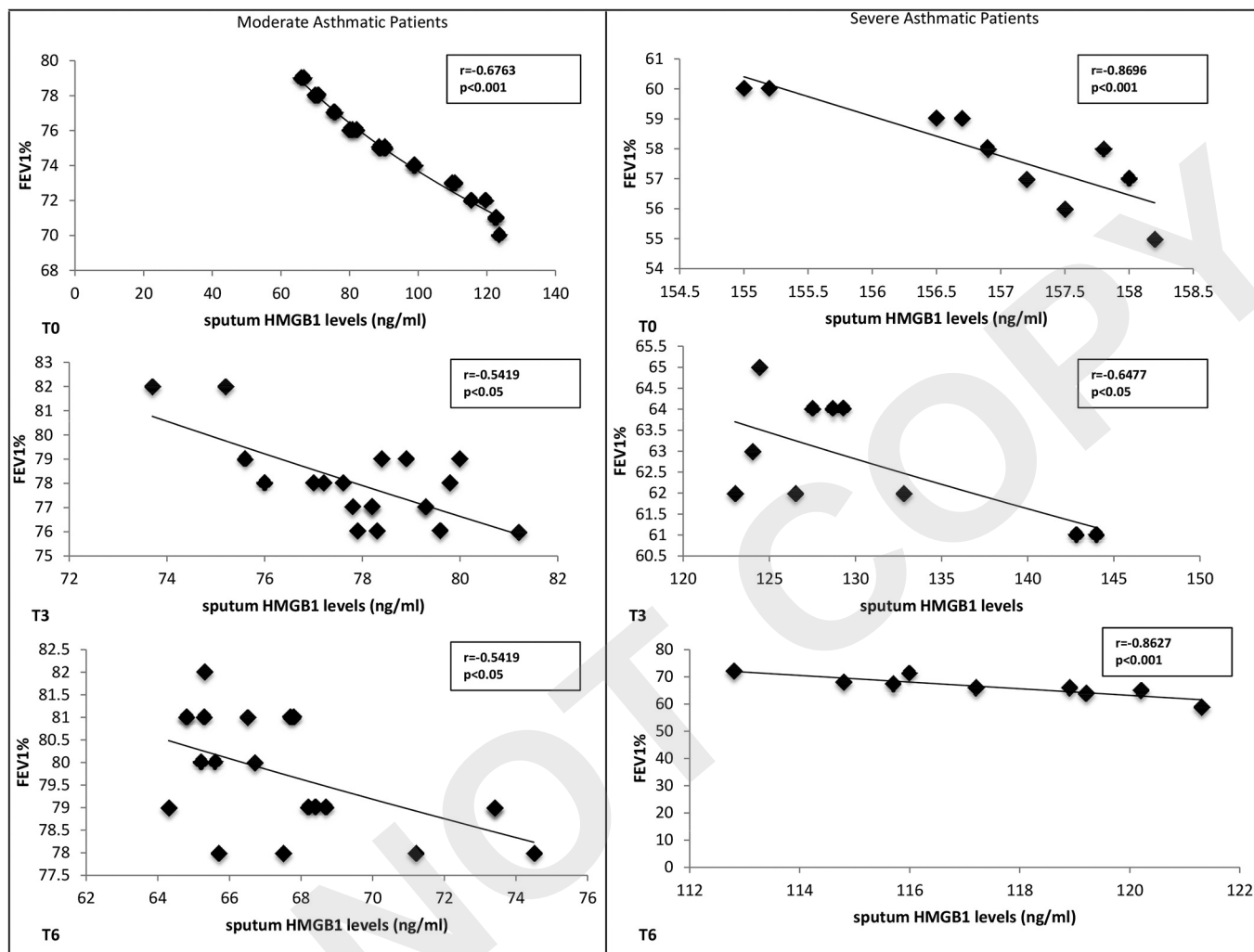


Figure 2. Correlations between sputum high mobility group box 1 (HMGB1) levels and percentage forced expiratory volume in 1 second ($FEV_1\%$) in children with moderate (baseline [T0], 3 months [T3], 6 months [T6]) and severe asthma (T0, T3, T6).

rolled. All the subjects completed the study and were considered in the final analysis. There were no significant differences between the asthma group and the healthy controls with regard to sex ($p = 0.12$), mean age ($p = 0.21$), body mass index ($p = 0.11$), and family history of atopy ($p = 0.80$). Sputum HMGB1 levels were significantly higher in patients with asthma compared with healthy controls ($p < 0.0001$) (Fig. 1). In particular, patients with severe asthma reported higher sputum HMGB1 levels than patients with moderate asthma, 156.9 ± 1.09 versus 93.44 ± 20.65 ng/mL respectively; $p < 0.0001$). Furthermore, total serum IgE levels in the asthma group were significantly higher than the healthy controls ($p < 0.0001$) and were even positively related to sputum HMGB1 values ($r = 0.6567$; $p < 0.0001$). The data are summarized in (Table 1).

HMGB1 and Lung Function at T0, T3, and T6

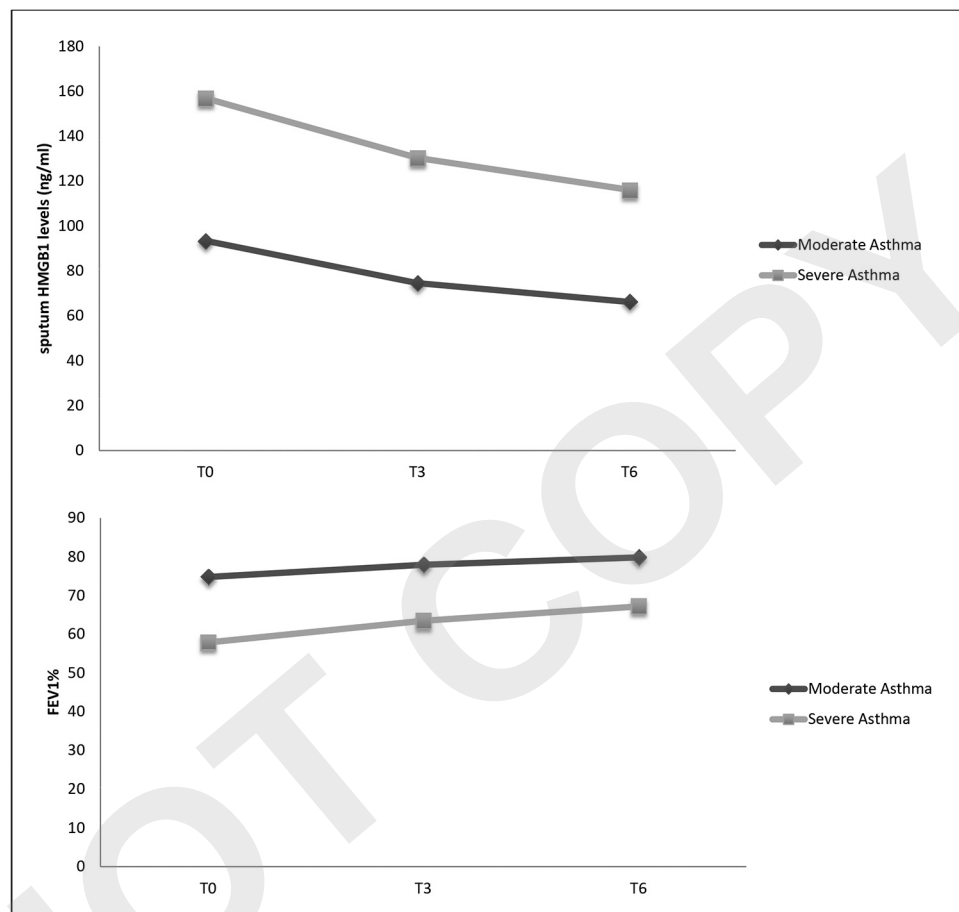
An inverse and significant correlation between sputum HMGB1 levels and the pulmonary function index,

such as the $FEV_1\%$, was observed in the children with moderate asthma (T0: $FEV_1\%$, $r = -0.9891$, $p < 0.001$; T3: $FEV_1\%$, $r = -0.6763$, $p < 0.001$; T6: $FEV_1\%$, $r = -0.5419$, $p < 0.05$) and in the children with severe asthma (T0: $FEV_1\%$, $r = -0.8696$, $p < 0.001$; T3: $FEV_1\%$, $r = -0.6477$, $p < 0.05$; T6: $FEV_1\%$, $r = -0.8627$, $p < 0.001$) (Fig. 2).

HMGB1 and Duration Treatment at T0, T3, and T6

After 3 months of ICS treatment, a significant decrease of sputum HMGB1 levels was noted in the children with moderate asthma (T3 [77.96 ± 1.81 ng/mL] versus T0 [93.44 ± 20.65 ng/mL]; $p < 0.0001$), and in the children with severe asthma (T3 [156.9 ± 1.09 ng/mL] versus T0 [130.3 ± 7.48 ng/mL]; $p < 0.0001$) (Fig. 3 a). After 6 months of ICS treatment, a further significant decrease of sputum HMGB1 levels was shown in the children with moderate asthma (T6 [67.75 ± 3.01 ng/mL] versus T3 [77.96 ± 1.81 ng/mL]; $p < 0.0001$) and in the children with severe asthma (T6

Figure 3. Sputum high mobility group box 1 (HMGB1) levels (a) and percentage forced expiratory volume in 1 second (FEV₁%) index (b) after 3 and 6 months of inhaled corticosteroid (ICS) treatment in patients with moderate asthma and patients with severe asthma.



[116.08 ± 4.77 ng/mL] versus T3 [130.3 ± 7.48 ng/mL]; $p < 0.0001$) (Fig. 3 a).

Lung Function and Duration Treatment at T0, T3, and T6

After 3 months of ICS treatment, a significant increase of FEV₁% was noted in the children with moderate asthma (T3 [77.95%] versus T0 [74.8 ± 6.36%]; $p < 0.05$) and in the children with severe asthma (T3 [62.8 ± 1.39%] versus T0 [57.9 ± 2.12%]; $p < 0.0001$) (Fig. 3 b). After 6 months of ICS treatment, a further significant increase of FEV₁% was shown in moderate (T6 [79.85 ± 1.41%] versus T3 [77.95%]; $p < 0.0001$) and in the children with severe asthma (T6 [67.2 ± 4.34%] versus T3 [62.8 ± 1.39%]; $p < 0.0001$) (Fig. 3 b).

Sputum HMGB1 Level as a Diagnostic Marker of Asthma Disease

With regard to pretreatment, the areas under the ROC curve were 0.705 and 0.728 for patients with moderate and those with severe asthma, respectively (Fig. 4 a and b). With regard to posttreatment, the areas under the ROC curve were 0.737 and 0.734 for patients with moderate asthma and those with severe asthma, respectively (Fig. 4, c and d).

DISCUSSION

The aim of this study was to investigate the role of HMGB1 as a novel inflammatory biomarker that could be used as guidance for management of children with asthma. Allergic asthma results from a complex interplay of genetic predisposition, environmental triggers, and epigenetic changes.^{1,2} Although current concepts of pathogenesis of asthma are focused on exaggerated T-helper cell type 2 airway inflammation and airway remodeling, HMGB1 also could play a pivotal role in the pathogenesis of asthma, both by inducing T-helper cell type 2 and eosinophilic and neutrophilic responses.^{14,16,17} The critical role of HMGB1, a new marker of inflammation that belongs to the alarmins family and in promoting an immediate immune response to tissue damage,¹⁸ has been assessed in several disorders.^{19–21} Particularly, after its release in the extracellular space, HMGB1 acts as a damage-associated molecular pattern molecule, initiating and perpetuating immune response both in infectious and noninfectious inflammatory processes.²² Its role, in fact, is to act as a “danger signal,” which orchestrates a homeostatic defensive response in challenged tissues.^{23,24}

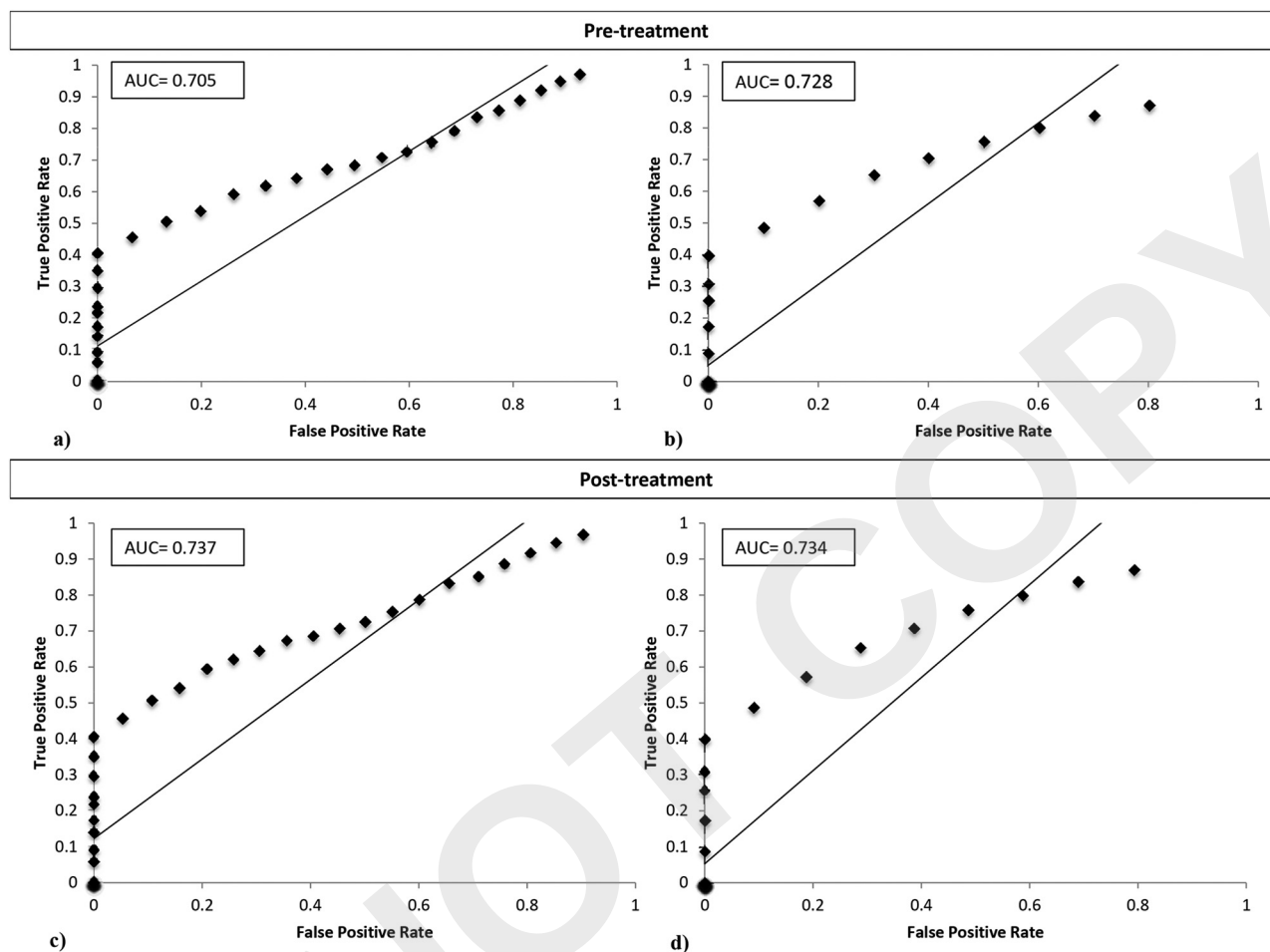


Figure 4. Receiver operating characteristic (ROC) curve analysis in patients with moderate (a, c) and patients with severe (b, d) asthma before and after treatment.

Thus, the recent discovery of HMGB1, as a critical mediator of inflammation, stimulated an increasing interest in the field of inflammation research, which indicates a new target disease. Therefore, although data in the literature are controversial concerning respiratory diseases, several studies highlight the strong relationship among asthma, lung function parameters, and HMGB1,^{6,9,10,25} which justify the increased HMGB1 levels in induced sputum and plasma reported in patients with asthma.^{26,27} Previously, we also assessed the role of this new inflammatory marker in children with allergic asthma.¹¹ In this study, we confirmed again that sputum HMGB1 levels are higher in children with allergic asthma than in healthy controls. Moreover, we also reported a direct correlation between sputum HMGB1 levels and total serum IgE levels, and an inverse relationship with lung function.

In line with the above-mentioned data, the findings described in the present report, to the best of our knowledge, represented the first description of the use of HMGB1 not only as a predictive marker of disease susceptibility but also as a marker of effectiveness of

ICS treatment of allergic asthma. In particular, sputum HMGB1 levels recorded after 6 months of therapy were significantly lower than those noted at T3. In parallel, sputum HMGB1 levels were inversely correlated to FEV1% and the lung function showed a significant increase at T6 compared with T3. However, as shown by pre- and posttreatment ROC curves, HMGB1 seemed to be a moderate diagnostic marker for this condition. Nevertheless, because we conducted a single-center study, the cohort of patients was relatively small to have a strong statistically significant coefficient.

CONCLUSION

Also, although we recognized that there were a number of limitations to our study, including the small number of the participants as well as the narrow age range, we also believe that our study provided further evidence that sputum HMGB1 is a sensitive biomarker to distinguish moderate from severe types of allergic asthma in children, and it seems to reflect obstructive disease status because there is an inverse correlation with lung function.

Moreover, sputum HMGB1 levels seem to also be useful tools to evaluate the response to ICS treatment because, in a time-dependent manner, the prolonged therapy significantly reduces sputum HMGB1 levels. Therefore, in light of these findings, we hypothesized that, by longitudinal HMGB1 measurement, it should be possible to monitor the persistent allergic minimal inflammation of the airway and, consequently, to provide further valuable information for the treatment duration.

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