

# Nasal High-Mobility Group Box-1 Protein in Children with Allergic Rhinitis

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## Key Words

Allergic rhinitis · High-mobility group box-1 protein · Nasal lavage

## Abstract

**Background:** Allergic rhinitis (AR) is characterized by an inflammatory reaction. High-mobility group box-1 protein (HMGB1) has many characteristics similar to classic proinflammatory cytokines. No study has yet investigated its role in AR. The aim of this study was to measure HMGB1 levels in the fluid recovered from nasal lavage in children with untreated AR and in control subjects. **Materials:** The study was conducted on 104 AR subjects (48 males and 56 females, median age  $10.3 \pm 3.4$  years) and 97 healthy children (42 males and 55 females) who were age-matched (median age  $9.8 \pm 4.1$  years). Total serum immunoglobulin E, peripheral eosinophils and nasal symptoms assessed by visual analog scale (VAS) were considered. HMGB1 was measured using an ELISA assay. **Results:** HMGB1 levels in nasal lavage fluid were higher in AR children than in the control group ( $96.9 \pm 19.3$  vs.  $9.27 \pm 4.01$  ng/ml;  $p < 0.001$ ). There was a very strong relationship between HMGB1 levels and VAS values in AR children ( $r = 0.919$ ). Considering the symptom severity assessed by VAS, there was a relationship between HMGB1 and VAS in

all AR subgroups: more evident in the severe subgroup ( $r = 0.727$ ). **Conclusions:** Nasal HMGB1 has significantly increased in children with AR and is significantly related to symptom severity.

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## Introduction

Allergic rhinitis (AR) is a common immunological disorder and is characterized by an immunoglobulin E (IgE)-mediated inflammation induced by allergen exposure. Infiltrating cells, including T cells, eosinophils, mast cells and basophils, release several mediators, which cause symptoms, and cytokines, that promote and amplify the inflammatory cascade.

Proinflammatory mediators play an important role in developing the persistence of nasal inflammation [1].

Recent studies have demonstrated that apoptotic and necrotic cells release damage-associated molecular pattern (DAMP) molecules, such as high-mobility group box-1 protein (HMGB1), capable of promoting early innate and adaptive immune responses through their interaction with pattern recognition receptors [2]. DAMPs contribute to the induction of inflammation by activa-

tion and recruitment of various inflammatory cells. Some studies have suggested that DAMPs also play an important role in the pathogenesis of allergic disease [3].

HMGB1, one of the most important DAMPs, has been discovered to have many characteristics similar to classic proinflammatory cytokines [4]. HMGB1 is a bifunctional protein that has completely distinct functions in nuclear and extracellular environments. In the nucleus, this protein binds DNA in a nonspecific manner and induces substantial distortion of DNA, which is involved in gene regulation (DNA replication and transcription) [5]. On the other hand, it has been recognized that HMGB1 can be actively secreted into the extracellular space by activated monocyte/macrophages [6, 7]. Several studies have provided evidence that extracellular HMGB1 is involved in a variety of pathophysiological events, including diabetes [8], antibacterial activity [9], smooth-muscle-cell chemotaxis [10], cell differentiation [11, 12], myocardial regeneration [13], angiogenesis [14], tissue repair [15] and cancer [16]. So HMGB1 acts as potent proinflammatory cytokine [17, 18].

HMGB1 is a potent stimulator of tumor-necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , IL-8, monocyte chemoattractant protein-1 and plasminogen activator inhibitor-1 secretion. HMGB1 also interacts with some receptors, such as RAGE, toll-like receptor 2 (TLR2), TLR4 and TLR9, to induce various cellular responses [19–21]. HMGB1 increases the expression of adhesion molecules in the endothelium, including ICAM-1 and VCAM-1 [22].

Recently, Watanabe et al. [23] showed that in asthmatic patients, HMGB1 levels were significantly correlated with the percentage of neutrophils in induced sputum, and proposed this as a marker of neutrophilic inflammation. In contrast, Shim et al. [24] showed a correlation of HMGB1 with sputum eosinophilia. More recently, a preliminary pilot study showed that the HMGB1 level had increased in the nasal mucus of 35 children with AR and both intranasal corticosteroid and glycyrrhetic acid were able to diminish them [25]. Moreover, it has been previously reported that the HMGB1 increased in the plasma and sputum of patients with asthma and chronic obstructive pulmonary disease, in comparison with normal controls, and there was a significantly negative correlation with the pulmonary function index [26]. However, there is no study that has investigated HMGB1 in AR. We hypothesized that HMGB1 might be a player in the inflammatory scenario of AR. To test our hypothesis, we measured HMGB1 levels in the fluid recovered from nasal lavage from children with untreated AR and from control subjects.

## Materials and Methods

### *Subjects and Experimental Design*

This study included children with AR as well as healthy nonallergic children, recruited from 4 Italian pediatric departments (in Messina, Catania, Naples and Pavia). All the participating subjects were of Italian origin. The study was approved by the ethical committee of the Policlinico of Messina (approval No. 137, 31/01/2012). The study was explained in detail to the parents/guardians of the children who participated and an informed written consent was obtained from each parent/guardian. A physician performed the physical examination of each child. Demographic data and other clinical characteristics of the study subjects were collected using the questionnaires.

AR was diagnosed according to the presence of typical nasal symptoms (nasal itching, sneezing, watery rhinorrhea and nasal obstruction) and sensitization to pollen allergen, documented by a positive skin prick test (SPT) [27]. Inclusion criteria were: age of between 6 and 16 years, a diagnosis of AR, sensitization to *Parietaria* pollen and the presence of nasal symptoms.

Exclusion criteria were: perennial AR, any respiratory tract infection within 1 month before the start of the study, other nasal disease (including structural abnormalities, nasal polyposis and clinically relevant septum deviation), asthma comorbidity (excluded by history and physical examination) and any treatment in the previous month capable of interfering with the results.

Healthy subjects had to have: no history or symptoms of AR, asthma or other allergic disorders, a negative SPT and total serum IgE levels below the general population mean. Controls were sampled at the same time.

The study was carried out from March to May 2012, this being the time during which subjects were naturally exposed to pollens. It was conducted on 104 AR subjects (48 males and 56 females, median age  $10.3 \pm 3.4$  years). All children presented with: nasal obstruction, nasal itching, sneezing and watery rhinorrhea, an increased level of total serum IgE and positive results for the *Parietaria* pollen SPT.

The control group consisted of 97 age-matched, healthy children (42 males and 55 females; median age  $9.8 \pm 4.1$  years) with a negative history of allergic disease, a normal level of total serum IgE and negative results for the SPT. The SPT was performed using a panel of aeroallergens, including house-dust mite, mixed grass, *Parietaria*, birch, olive tree, *Alternaria*, cat and dog; Allergopharma, Reinbek, Germany). Normal saline and histamine were used as negative and positive controls, respectively. Skin wheal diameter was recorded at 15 min as the mean of 2 perpendicular measurements. A positive response was defined as a skin wheal diameter of 3 mm or more compared to a negative control.

The severity of nasal symptoms was assessed using the Visual Analog Scale (VAS). The patients had to indicate nasal symptom severity via the VAS by putting a mark on a 10-cm segment. At the left end of the line (0 cm) symptoms were absent and at the right end (at 10 cm) severity was maximal [28]. VAS referred to the symptoms perceived during the past week.

Based on the symptom severity, we subdivided the AR children into 3 groups: (1) mild severity characterized by VAS value  $<5$ , (2) moderate severity by VAS value  $\geq 5$  and  $<8$  and (3) severe severity by VAS value  $\geq 8$  [29].

All subjects underwent blood sampling and nasal lavage. The blood samples from the healthy controls and AR subjects were

collected by the vein-puncture method under appropriate conditions. The sample were collected and stored at  $-20^{\circ}\text{C}$  until the protein measurement was made.

To collect the fluid recovered from nasal lavage, each child was placed in a sitting position with the head tilted slightly. The nasal lavage was performed with a pipette, injecting 1.5 ml of saline solution (0.9% NaCl at  $37^{\circ}\text{C}$ ) into the nostril of the child, after closing the possible escape route represented by the other nostril with a patch. The recovered liquid was collected after a few seconds with the same pipette. The sampling was performed for both nostrils. The fluid was stored at  $-20^{\circ}\text{C}$  until the measurement was made. The recovery volume was on average more than 90% after nasal lavage.

Total serum IgE and peripheral eosinophil levels were determined in all subjects. Total IgE was measured by ImmunoCAP100 system (Phadia, Uppsala, Sweden).

#### HMGB1 Measurement

The nasal HMGB1 concentration was measured by performing an enzyme-linked immunosorbent assay using an HMGB1 ELISA Kit II (IBL by Shino-Test Corporation; Kanagawa, Japan). The limit detection was  $0.2\text{ ng/ml} + 2.6\text{ SD}$  and the limit of quantification was  $0.1\text{ ng/ml}$  with a coefficient of variation of  $\leq 20\%$  for the high sensitive range.

The samples were diluted 1:5 in sample diluent and added to microtiter plates, which were then incubated for 24 h at  $37^{\circ}\text{C}$ . After washing,  $100\text{ }\mu\text{l}$ /well of anti-human HMGB1 peroxidase-conjugated monoclonal antibody were added and the plates were incubated at room temperature for 2 h. After washing, 3,3',5,5'-tetramethylbenzidine dihydrochloride was added to each well. The enzyme reaction was allowed to proceed for 30 min at room temperature. The chromogenic substrate reaction was stopped by the addition of stop solution (0.35 mol/l 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  $0.2\text{ ng/ml}$ .

#### Data Analysis

Statistical analysis was performed with an ANOVA test and relatives post hoc versus control. Mean and standard deviation were calculated for the variables and a Student t test was employed for the comparisons. For the attributes, percentages were calculated first and then a  $\chi^2$  test was used for comparisons. The data were analyzed by the statistical computer software SPSS, version 15.0 (SPSS Inc, Chicago, Ill., USA). A p value of  $<0.05$  was considered to be statistically significant.

## Results

The characteristics of the 2 groups are presented in table 1. Age and gender did not significantly differ between groups. Serum total IgE and peripheral eosinophils were significantly increased in allergic children in comparison with healthy children ( $p < 0.01$  for both).

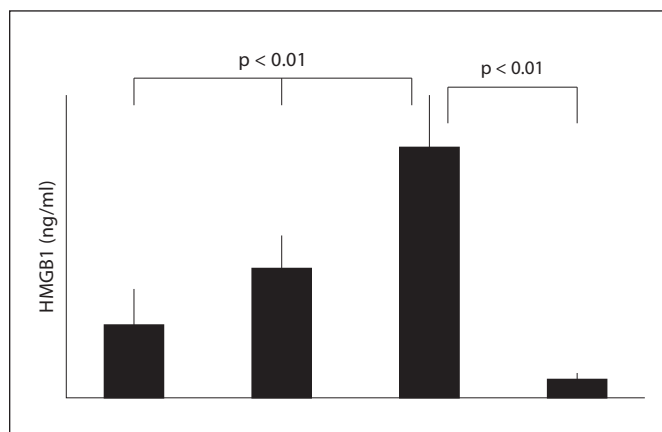


**Fig. 1.** Nasal HMGB1 levels in children with AR, considering the symptom severity (mild, moderate and severe) and in healthy children.

**Table 1.** Characteristics of the subjects

|                                 | Rhinitic subjects | Control subjects | p value |
|---------------------------------|-------------------|------------------|---------|
| Number of subjects              | 104               | 97               |         |
| Age, years                      | $10.3 \pm 3.4$    | $9.8 \pm 4.1$    | 0.422   |
| Male/female                     | 48/56             | 42/55            | 0.121   |
| Serum total IgE, IU/ml          | $199.1 \pm 29.69$ | $39.75 \pm 6.25$ | 0.01    |
| Eosinophil count, $\text{mm}^3$ | $419.8 \pm 99.7$  | $137 \pm 17.9$   | 0.01    |

Data are expressed as means  $\pm$  SD.

In addition, 37 children had mild rhinitis ( $\text{VAS } 3.5 \pm 1.5$ ), 32 had moderate rhinitis ( $\text{VAS } 6.5 \pm 1.5$ ) and 35 had severe rhinitis ( $\text{VAS } 9.5 \pm 1.5$ ) as shown in table 2. There was a significant distribution of serum total IgE levels in the 3 AR subgroups as well as the eosinophil count being higher in children with more severe symptoms ( $p < 0.01$  for both parameters as reported in table 2).

HMGB1 levels in nasal lavage fluid were higher in children with AR than in the control group ( $96.9 \pm 19.3$  vs.  $9.27 \pm 4.01\text{ ng/ml}$ ;  $p < 0.001$ ).

The highest levels of HMGB1 were found in the group of children with severe symptoms and there was a significant difference among AR subgroups (table 2; fig. 1).

There was no relationship between HMGB1 levels and serum total IgE levels or between HMGB1 and eosinophil count (fig. 2a, b). On the other hand, there was

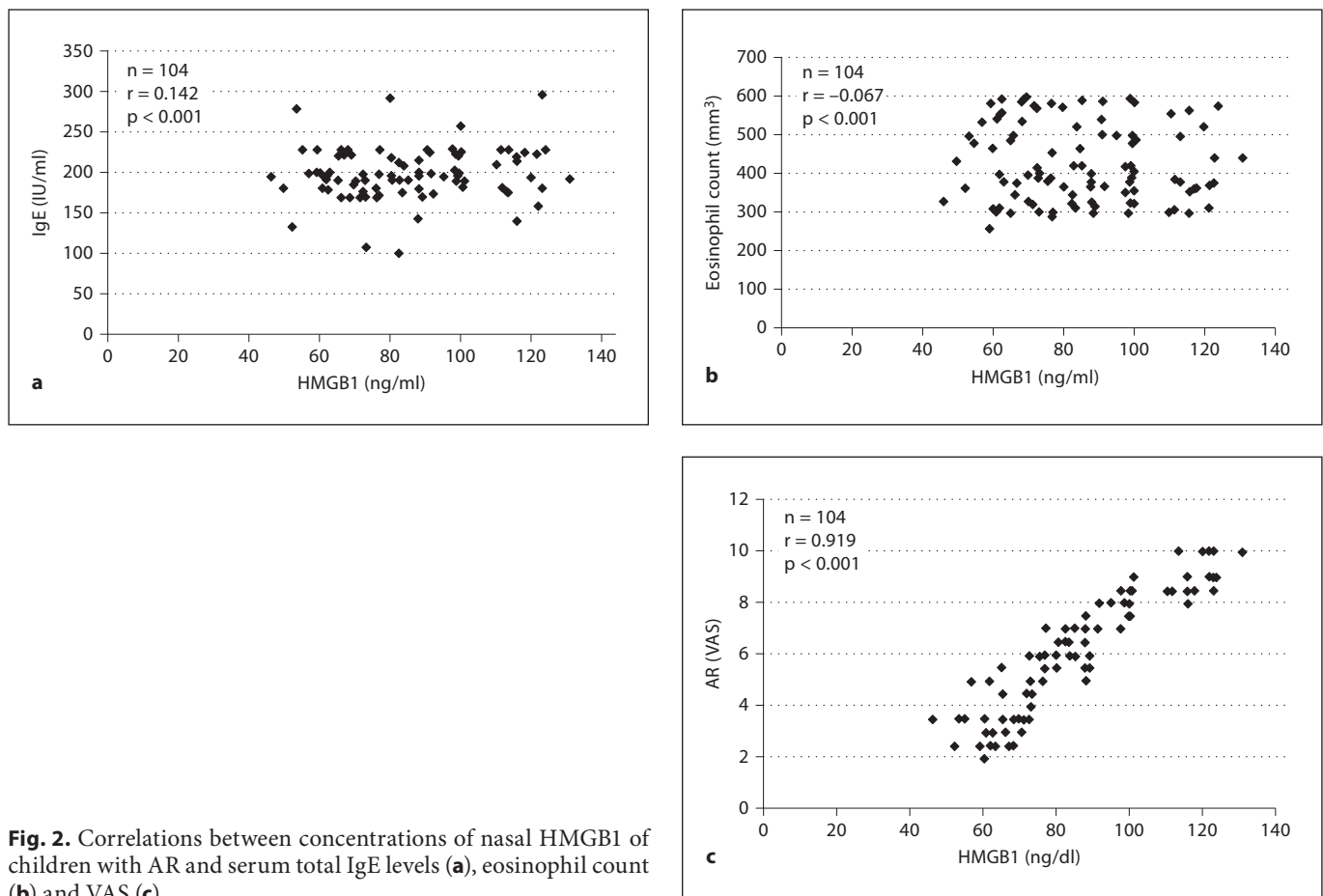


**Fig. 2.** Correlations between concentrations of nasal HMGB1 of children with AR and serum total IgE levels (**a**), eosinophil count (**b**) and VAS (**c**).

**Table 2.** Concentration of HMGB1 in nasal lavage, serum total IgE and peripheral eosinophil count in patients with AR (of different severities) and in healthy children

|                                   | 37 Mild<br>(VAS 3.5 ± 1.5) | 32 Moderate<br>(VAS 6.5 ± 1.5) | 35 Severe<br>(VAS 9.5 ± 1.5) | Control<br>group |
|-----------------------------------|----------------------------|--------------------------------|------------------------------|------------------|
| HMGB1, ng/ml                      | 34.39 ± 17.09              | 61.06 ± 15.62                  | 118.27 ± 24.74               | 9.27 ± 4.01      |
| Total IgE, IU/ml                  | 73.7 ± 12.5                | 125.4 ± 14.3                   | 178.8 ± 32.3                 | 39.75 ± 6.25     |
| Eosinophil count, mm <sup>3</sup> | 325 ± 22.3                 | 443.2 ± 32.3                   | 563.3 ± 12.3                 | 137 ± 17.9       |

a very strong relationship between HMGB1 levels and VAS values in AR children ( $r = 0.919$ ), as reported in figure 2c. Considering the symptom severity assessed by VAS, there was a relationship between HMGB1 and VAS in all AR subgroups: more evident in the severe subgroup ( $r = 0.727$ ). In addition, there was a significant relationship between both serum total IgE levels and eosinophils with VAS values ( $r = 0.62$  and  $r = 0.53$ , respectively).

## Discussion

Nasal symptoms depend on both the release of mediators (typically histamine) and local inflammation (mainly characterized by eosinophil infiltrate). In this scenario, a cascade of events occurs and several proinflammatory molecules play a pathogenic role.

Extracellular HMGB1 acts as a proinflammatory cytokine [4, 11] and stimulates neighboring cells, including

endothelial cells, to express proinflammatory cytokines, chemokines and adhesion molecules. These mediators activate infiltrating macrophages/monocytes and endothelial cells, thereby increasing the release of HMGB1 and other mediators. Therefore, HMGB1 (by its cytokine-like activity) contributes to the pathogenesis of inflammatory phenomena, amplifying the cascade of events. However, there is no study that has yet investigated its role in AR.

Our study aimed at evaluating the possible role of HMGB1 in children with AR due to *Parietaria* pollen sensitization.

Firstly, it has been demonstrated that children with AR have higher HMGB1 concentrations than nonallergic subjects. Moreover, there is a significantly increasing trend related to symptom severity. In fact, patients with the most severe symptoms have the highest HMGB1 serum levels. To confirm this relationship, a very strong association was globally evidenced between symptom severity and HMGB1. This finding remained when the patients were divided into 3 subgroups.

Secondly, both total IgE serum levels and peripheral eosinophil count were related to VAS values. These outcomes indirectly confirm the relationship between symptom severity assessed by VAS and surrogate markers of allergy severity, such as IgE and eosinophil.

It must be underlined that the measurement of HMGB1 was performed directly at the level of the target organ, the nose, i.e. this finding reflects the local inflammatory reaction.

On the other hand, Watanabe et al. [23] showed that HMGB1 levels were significantly correlated with the percentage of neutrophils in the induced sputum of asthmatic patients and proposed this as a marker of neutrophilic inflammation. In contrast, Shim et al. [24] showed a correlation between HMGB1 and sputum eosinophilia. Unfortunately, our study did not consider local markers of nasal inflammation. Another limitation is that there was no follow-up concerning the re-evaluation outside of the pollen season, so the real origin of HMGB1 is still obscure at the nasal level. More studies have to be performed regarding the pathway of HMGB1 in AR at the local level (origin, functionality and response to treatment) and its concentration outside of the pollen season in seasonal and perennial AR.

In conclusion, nasal HMGB1 has significantly increased in children with AR and is significantly related to symptom severity, and thus merits more attention in future research.

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