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# Increase in eosinophil-derived neurotoxin level in school children with allergic disease

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## ABSTRACT

**Background:** Eosinophils are major effector cells of allergic disease and excellent markers of eosinophilic inflammation. Accurate and reliable biomarkers are helpful in the diagnosis, treatment, and control of allergic disease.
**Objective:** This study aimed to investigate an alternate marker of eosinophilic inflammation, eosinophil-derived neurotoxin (EDN), in a number of allergic diseases.
**Methods:** Three hundred ninety-six elementary school-age children with various allergic conditions were recruited for this study. Subgroups included food allergies (FAs), atopic dermatitis (AD), bronchial asthma (BA), and allergic rhinitis (AR). EDN levels in these groups were compared to those in 93 healthy controls (HC).
**Results:** All subjects with allergic disease had elevated levels of serum EDN (median [interquartile range]: FA, 124.2 ng/mL [59.13–160.5 ng/mL]; AD, 110.8 ng/mL [57.52–167.9 ng/mL]; BA, 131.5 ng/mL [60.60–171.0 ng/mL]; AR, 91.32 ng/mL [46.16–145.0 ng/mL]) compared to HC (38.38 ng/mL [32.40–55.62 ng/mL]) ($p < 0.0001$). These elevated levels were consistent throughout the age range (6–12 years) of the healthy study subjects ($p = 0.0679$). EDN levels also correlated well with total immunoglobulin E (Rs = 0.5599, $p < 0.0001$). Looking at all individuals with an allergic disease, the area under the curve was 0.790.
**Conclusions:** Direct measures of eosinophilic inflammation are needed for accurate diagnosis, treatment, and monitoring of allergic diseases. EDN may be a worthy biomarker of eosinophil activity and a useful screening tool for allergic diseases including FA, AD, BA, and AR.

**Keywords:** Food allergy; Atopic dermatitis; Bronchial asthma; Allergic rhinitis; Biomarkers; Eosinophil-derived neurotoxin

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**Conflict of Interest**
The authors have no financial conflicts of interest.

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## INTRODUCTION

Eosinophils are major effector cells of the allergic process [1] and have proven to be the best marker of allergic inflammation. Activation of eosinophils leads to extracellular release of a number of granule proteins, such as eosinophil-derived neurotoxin (EDN) [2], and it is these granule proteins that have been the most strongly implicated in the pathophysiology of asthma [3].

EDN levels have been demonstrated to be an alternative measurement of the eosinophilic inflammation found in allergic disease [4-6]. It has been proposed that serum levels of degranulation products like EDN represent both the circulating levels and secretory activity of eosinophils [7]. Furthermore, several research groups have found EDN to be a more useful biomarker for evaluating disease severity than other degranulation products [8-10].

In this study, we aimed to compare EDN levels in a number of different groups of elementary school children with various allergic diseases: specifically, bronchial asthma (BA), atopic dermatitis (AD), allergic rhinitis (AR), and food allergy (FA).

## MATERIALS AND METHODS

### Subjects

Three hundred ninety-six elementary school-age children were recruited from an elementary school attached to Chiba University. Details of control subjects have been described previously [11] and can be found in **Table 1** of this study. The study protocol was approved by the Committee on Human Research of Chiba University (IRB number: 0710). Informed consent was obtained from each of the study subjects and their guardians. BA diagnosis was made according to the ISAAC (International Study of Asthma and Allergies in Childhood) questionnaire. Concurrent physician-diagnosed AD, AR, and FA were assessed. Of the 250 children with any type of allergic disease, 27.6% had multiple allergic diseases. Children with no allergic disease and those not exhibiting serum specific immunoglobulin E (IgE) to house dust mites, cedar pollen or egg whites were defined as healthy children.

Inclusion criteria for allergic diseases included symptomatic or asymptomatic asthma or allergic diseases. Exclusion criteria included: any health condition that would affect the ability of the patient to give a blood specimen; or any potential for serious adverse events. None of the healthy children had a history of asthma or allergic diseases.

**Table 1.** Baseline characteristics of subjects

| Characteristic | Healthy controls (n = 93) | Four allergic disease subgroups | | | |
|---|---|---|---|---|---|
| | | Food allergies (n = 34) | Atopic dermatitis (n = 34) | Bronchial asthma (n = 39) | Allergic rhinitis (n = 143) |
| Age (yr) | | | | | |
| Mean ± SD | 8.9 ± 1.7 | 9.0 ± 1.8 | 9.4 ± 1.6 | 9.3 ± 1.8 | 9.5 ± 1.7 |
| Median (range) | 9 (6–12) | 9 (6–12) | 10 (7–12) | 9 (7–12) | 10 (7–12) |
| Sex (male %) | 36.30 | 50.00 | 54.20 | 69.20 | 60.80 |
| Serum IgE (IU/mL) | | | | | |
| Mean ± SD | 34.8 ± 54.6 | 473.5 ± 683.9 | 478.7 ± 608.1 | 346.1 ± 304.2 | 308.6 ± 3,329.3 |
| Median (range) | 21.2 (5.0–108.0) | 153.0 (5.8–2,150.0) | 378.0 (13.2–1,160.0) | 221.0 (29.1–1,080.0) | 184.0 (7.5–1,020.0) |

SD, standard deviation; IgE, Immunoglobulin E.


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### Blood specimen collection

BD Vacutainer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) serum separation tubes were used to collect blood specimens. The tourniquet was removed from the arm as soon as blood flowed to prevent hemoconcentration. Care was taken to perform venipuncture in a manner so that the likelihood of any complication following this was minimized. The nurse performing the venipuncture observed universal precautions for the prevention of bloodborne pathogen transmission.

### Serum EDN measurement

Serum specimens were prepared, as described by Peterson et al. [12]. Briefly, serum was prepared by allowing blood to clot at 25°C for 1 hour, then centrifuged at 1,350 g for 10 minutes at 4°C. Each serum specimen was aliquoted into a new plastic tube and stored at -70°C until the assay.

The central laboratory at Inje University Sanggye Paik Hospital in Seoul, Korea was used for serum EDN (sEDN) measurements. sEDN concentrations were measured using the K®EDN "sandwich" enzyme-linked immunosorbent assay (ELISA) [13] kit (SKIMS-BIO Co., Seoul, Korea), with results expressed in ng/mL. This ELISA detects human EDN with a minimum detection limit of 6.0 ng/mL, maximum detection limit of 400 ng/mL, and does not cross-react with eosinophil cationic protein (ECP). The method described by Morioka et al. [14] was followed but modified slightly. Briefly, Nunc MaxiSorp 96-well plates (Thermo Fisher Scientific, Waltham, MA, USA) were coated overnight at 4°C with 100 µL of mouse anti-EDN monoclonal antibody (mAb), diluted in phosphate-buffered saline (PBS). The wells were blocked overnight at 4°C with 200 µL of blocking buffer (1X PBS, 1% bovine serum albumin [BSA], 10% sucrose). Standard EDN was diluted with 50 mM tris pH 8.0 containing 0.05% Tween 20 buffer (Sigma-Aldrich, St. Louis, Mom USA), 0.15 M NaCl, and 0.5% BSA (termed assay diluent). The range of measurements was 0.6–40 ng/mL, indicating assay sensitivity was less than 0.6 ng/mL. Between each subsequent step, plates were washed 3 times in PBS containing 0.05% Tween 20. Samples were then diluted in 50 mM tris pH 8.0 containing 0.05% Tween 20, and 0.15 M NaCl. One-hundred microliter of standards and diluted samples were applied to the plates, and incubated at room temperature for 1 hour. After washing, 100 µL of horseradish-peroxidase-labeled mouse anti-EDN mAb was added to the wells and incubated at room temperature for 1 hour. After another washing, the peroxidase substrate tetramethylbenzidine (Sigma-Aldrich) was added (100 µL/well) and incubated for 10 minutes at room temperature. Enzyme reactions were stopped with 1N HCl (100 µL/well). Absorbance was measured at 450 nm by a Micro Plate Reader Infinite 200 PRO (TECAN, Männedorf, Switzerland). sEDN was determined from a dose response curve by multiplying the value read from the standard curve by the dilution factor.

### Statistical analysis

Data are represented as mean ± standard deviation (SD) if normally distributed, or median and range if not normally distributed. Differences in subject characteristics were compared across subgroups with the chi-squared test or Fisher exact test for categorical variables, and analysis of variance with Scheffe *post hoc* test or Kruskal-Wallis test with Dunn *post hoc* test for continuous variables, as applicable. Normality tests for data distribution were performed using Shapiro-Wilk test. Univariate and multivariate logistic regression analyses were performed to identify prognostic factors that were independently associated with prolonged hospitalizations. A multivariate model was created using a backward elimination method, and the probability was set at 0.05 for removal. Odds ratio with 95% confidence interval

were calculated for prognostic factors associated with prolonged hospitalizations. The cutoff values for EDN statistics were based on 45 ng/mL. All statistical analyses were performed with IBM SPSS Statistics ver. 24.0 (IBM Co., Armonk, NY, USA) and a $p$ value of <0.05 was considered statistically significant.

## RESULTS

### Subject characteristics

Baseline subject characteristics are displayed in **Table 1**. There were no significant differences in characteristics among the different groups.

### Serum levels of EDN

Serum levels of EDN are shown in **Fig. 1**. Median (interquartile range) for each group were as follows: healthy controls (HC), 38.38 ng/mL (32.40–55.62 ng/mL); FA, 124.2 ng/mL (59.13–160.5 ng/mL); AD, 110.8 ng/mL (57.52–167.9 ng/mL); BA, 131.5 ng/mL (60.60–171.0 ng/mL); AR, 91.32 ng/mL (46.16–145.0 ng/mL). Comparing EDN levels in HC to the other groups showed significant differences: HC vs. FA ($p$ < 0.0001); HC vs. AD ($p$ < 0.0001); HC vs. BA ($p$ < 0.0001); HC vs. AR ($p$ < 0.0001).

Subgrouping EDN levels according to age in healthy subjects in **Fig. 2**, median (interquartile range) were as follows: age 6, 21.93 (18.38–34.49); age 7, 37.84 (32.40–52.18); age 8, 44.07 (36.24–62.54); age 9, 38.26 (28.45–53.80); age 10, 41.46 (31.99–56.40); age 11, 35.33(28.16–67.72). There were no significant differences in serum EDN level among the age groups ($p$ = 0.0679).

There was good correlation (Rs = 0.5599, $p$ < 0.0001) between sEDN level and total IgE (**Fig. 3**).

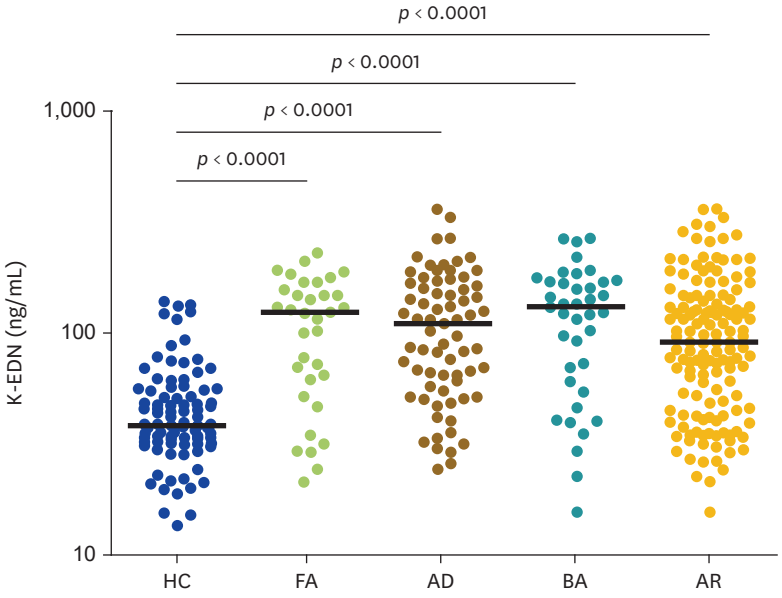


**Fig. 1.** Serum eosinophil-derived neurotoxin (EDN) levels in each study subgroup. Healthy controls (HC) exhibited significantly lower levels ($p$ < 0.0001) than all other subgroups with allergic disease: food allergy (FA), atopic dermatitis (AD), bronchial asthma (BA), and allergic rhinitis (AR).


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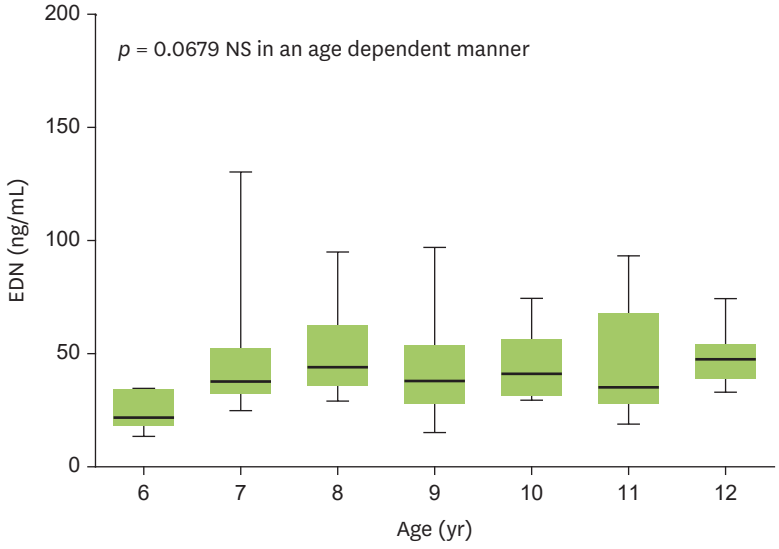


**Fig. 2.** Eosinophil-derived neurotoxin (EDN) level according to age. No significant difference ($p$ = 0.0679) in EDN level among all age groups.

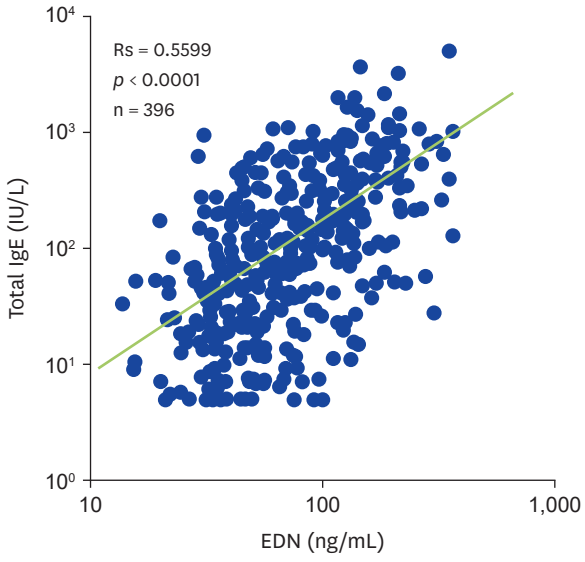


**Fig. 3.** Correlation between total immunoglobulin E (IgE) and eosinophil-derived neurotoxin (EDN) level (Rs = 0.5599; $p$ < 0.0001).

**Fig. 4** shows the area under the curve (AUC) for all allergic diseases in this study (i.e., FA, AD, BA, and AR). The true positive rate (aka sensitivity) (proportion of those patients with an elevated sEDN level that also have an allergic disease) was plotted on the *y* axis, while the false positive rate (aka 1 – specificity) (1 – proportion of those patients with a normal sEDN level that *do not* have an allergic disease) was plotted on the *x* axis. The AUC from an receiver operating characteristic curve analysis of serum EDN for all allergic diseases (FA, AD, BA, and AR) was 0.790 with 81.2% sensitivity and 69.8% specificity.


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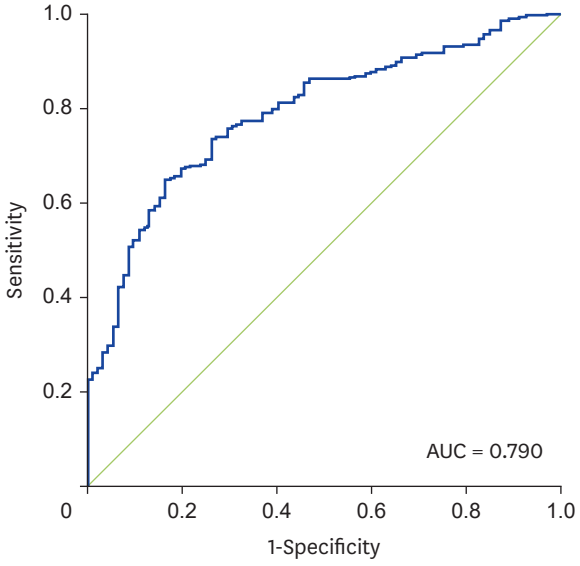


**Fig. 4.** Area under the curve (AUC) for all subjects with allergic disease as one group.

## DISCUSSION

Measuring sEDN levels in school-age children with physician-diagnosed allergic disease and comparing these levels to healthy children of the same age, we found significant elevation of EDN in those with disease. Adding our findings to those of previous studies on EDN and allergic disease, we can conclude that EDN is a good biomarker for eosinophilic inflammation and may be useful as a screening tool.

Diagnosis of allergic disease in young children is a difficult task. Assessment of symptoms and airway function have traditionally been used to diagnose, treat, and control allergic disease such as asthma [15, 16]. However, it is especially difficult to test airway function in young children because of their inability to participate in the various tests. Furthermore, the use of these traditional markers of airway inflammation is tenous due in part to the fact that airway function and symptoms can be temporarily improved by bronchodilators with no concomitant control of inflammation [17]. Persistent inflammation has been conclusively demonstrated in patients treated according to symptoms and lung function [18]. The sEDN levels measured in our test groups reflect this. Healthy children with no diagnosed allergic disease measured at a median of 38.38 ng/mL, significantly lower than levels found in children with FAs (124.2 ng/mL), AR (91.32 ng/mL), BA (131.5 ng/mL), or allergic dermatitis (110.8 ng/mL). In previous studies [10], our research group found EDN levels to be predictive for asthma. Using 44.2 ng/mL (median ± 1SD) as the cutoff for an elevated EDN level compared to those found in HC, sensitivity was 81.3%, specificity was 87.1%, positive predictive value was 90.7%, and negative predictive value was 75.0% [4]. It has been suggested that the secretory activity of eosinophils—a combination of concentration of eosinophils and their propensity to release degranulation products—may be a key marker of disease activity, and is more accurately measured by eosinophil degrnaulation products such as EDN [7].

In our study, we investigated sEDN levels in children from 6 to 12 years of age. When our study subjects were separated according to age, there was no significant difference in EDN level. This suggests that using EDN level as a biomarker for screening (i.e., diagnosis),


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treatment, and monitoring could be useful throughout childhood [19]. There have also been several studies of sEDN levels in children 6 and under and with a variety of conditions, including asthma, AD, AR, and FA. For example, a recently published study [5] of children aged 1 to 6 with asthma (beta2-agonist responsive recurrent and multiple trigger wheeze) and parental asthma or physician-diagnosed AD, AR, or FA found a majority of recruited subjects had elevated levels of sEDN (defined as ≥53 ng/mL). Furthermore, their EDN levels were used as a secondary outcome of treatment. Elevated sEDN levels in this age group have been found in other studies [4, 5, 10], as well. The utility of EDN as a biomarker in 6 and under children is especially important considering the difficulties of diagnosing, treating, and monitoring allergic disease in this age group.

As expected, total IgE correlated well with EDN level. IgE is one of the most important allergy biomarkers, as it is a major contributing factor in most types of allergic disease [20], including asthma, allergic dermatitis, AR, and IgE-mediated FA. Though asthma is a heterogeneous disease with many clinical subtypes, the classical IgE-associated asthma phenotype (also termed 'extrinsic') starting in childhood is the most widely studied, as it is the most common in real life and easily studied in the clinical laboratory [21]. As part of the 'one airway, one disease' hypothesis [22], it is believed rhinitis and asthma are 2 manifestations of a single syndrome, the chronic allergic respiratory syndrome, and it has been shown that one therapy can treat both diseases simultaneously [23, 24]. The correlation between the serum EDN level and the serum total IgE level is associated with an atopic status in elementary school-age children.

One of the key investigative goals of our study was to assess EDN as a biomarker for allergic activity in young children. As a group, EDN levels in our study subjects, who all had physician-diagnosed allergic disease, showed good sensitivity and specificity. It has been suggested by many that treatment based on inflammatory markers is more efficacious (i.e., reduces the frequency and severity of exacerbations) than treatment based on clinical symptoms and other traditional objective measures of lung function, such as peak expiratory flow and spirometry [25]. EDN is a more accurate reflection of eosinophil activity than merely counting eosinophil numbers, and EDN is a more easily recoverable degranulation product than ECP because ECP is sticky (i.e., sticks to tube walls) and a more highly charged protein [26].

Knowing both the inflammatory profile and the clinical categorization of the patient develops a clearer phenotype of the allergic disease patient and appears to contribute to improved care. Using a more accurate biomarker of eosinophilic inflammation, such as EDN, in disease management may produce even better results.

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