

Natural history of eosinophil-derived neurotoxin levels and the onset of allergic airway disease in preschool children

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Abstract. ‘Atopic march’ is the progression of allergic conditions through infancy and childhood. The present study investigated the association between blood eosinophil-derived neurotoxin (EDN) levels in preschool children with food allergy (FA) or atopic dermatitis (AD) and the onset of allergic airway disease [bronchial asthma (BA), allergic rhinitis (AR)]. A total of 123 children below the age of 1 year were enrolled in the present study, along with controls (n=37). Blood specimens were taken, serum EDN levels were measured and immunoglobulin E was quantified. Finally, a total of 86 subjects were analyzed. EDN values were measured at 3 time-points: before 1 year of age, before 2 years of age and before 3 years of age. The EDN levels were initially similar between those patients who did and those who did not develop allergic airway disease but then markedly diverged at the 2-year time-point (226.6 vs. 65.0 ng/ml; P<0.01) and remained divergent at the 3-year time-point (173.9 vs. 62.7 ng/ml; P<0.01). EDN levels prior to diagnosis were compared between the two groups and they were much higher in the Onset group (n=10) compared to the Non-onset group (n=67) (171.2±34.28 vs. 81.3±10.02 ng/ml; P=0.003), with 4 cases of BA and 6 cases of AR in the Onset group. After diagnosis, EDN levels were compared twice: i) At 1 and 2 years of age; and ii) 1 and 3 years of age. A significant difference was found only in the comparison at 2 years (P=0.001). In conclusion, young children with elevated EDN levels during the FA/AD disease period were more likely to develop allergic airway disease (BA, AR) in their first three

years of life. A factor leading to this progression may be increased eosinophil activity.

Introduction

The ‘atopic march’, at times referred to as the ‘allergic march’, is the progression of allergic conditions as they develop through infancy and childhood. These allergic conditions have common genetic and environmental predisposing factors, share immunologic features of one or more allergen-specific T helper type 2 responses and are characterized by a ‘type 2’ effector phase that includes the generation of specific Immunoglobulin E (IgE), granulocyte activation and other features, such as mucous production and edema. A key trait of the atopic march is that the presence of one allergic condition increases the risk for the development of others; hence the term ‘march’ (1). The atopic march typically begins with atopic dermatitis (AD) and then progresses to IgE-mediated food allergy (FA), asthma and allergic rhinitis (AR) (2).

The positive rate of specific IgE antibodies to food allergens, such as eggs, is high in infancy (3), and the positive rate of inhalant allergens (e.g., mites and house dust) increases with age. In addition, the prevalence of food allergies has increased in the past 30 years, particularly in industrialized countries (3). Among specific IgE measuring reagents, diagnostic reagents that may measure multiple items simultaneously are used for screening for sensitized allergens. MAST III and MAST IV (hereinafter referred to as ‘MAST’) are the most commonly available in Japan and Korea. MAST tests >33 specific IgE items simultaneously with a small amount of serum and is able to detect changes in specific antibody titers of multiple allergens in the atopic march during infancy. The results obtained may enable us to understand the tendency of allergy onset and provide preventive medical knowledge of future allergy risk.

The eosinophil is a major effector cell in allergic disease (4). Therefore, direct measurement of eosinophilic inflammation should aid in the diagnosis, treatment and monitoring of allergic disease. However, eosinophil counts/percentages provide only a limited understanding of eosinophil activity. It has been suggested that the secretory activity of eosinophils would be better measured by determining the concentration of eosinophils and their tendency to release mediators (5). In

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the past few decades, eosinophil-derived neurotoxin (EDN) has emerged as a promising biomarker for eosinophil activity and it has been studied in a number of inflammatory diseases, including asthma (6-10). EDN has been used successfully as a predictive marker for recurrent wheezing after respiratory viral infection (11), but there are few reports of using EDN levels as a predictor of airway allergic disease in preschool children.

In the present study, it was investigated whether there is an association between blood EDN levels and the onset of allergic disease in preschool children.

Subjects and methods

Subjects. Children of <1 year of age with food allergy disease and their controls who visited hospitals in Tochigi, Japan affiliated with the Tochigi Pediatric Allergy Study Group were enrolled. A total of 123 patients were initially enrolled in the study from March 2018 to December 2019, comprising 88 males and 35 females, along with 37 Controls.

The inclusion criteria were as follows: i) Male and female children <1 year of age at the time of enrolment; ii) children diagnosed with FA or AD; iii) those for whom written informed consent of their legal guardians was obtained. The diagnostic criteria for AD were 'chronic eczema lasting for >2 months, with other skin diseases excluded'. As controls, healthy children and those with non-allergic disease (children without allergic symptoms) were included (age- and sex-matched). These children were visiting well-baby clinics for vaccination. Even if all specific IgE levels were negative or there were no allergic symptoms, it was possible for allergies to develop during the clinical study period, in which case the final decision to include/exclude the subject was made at the end of the study.

Individuals to whom any of the following applied were excluded from the study: i) Patients with a history of cardiac, hepatic or renal disease who were undergoing treatment (excluding those receiving oral immunotherapy); ii) patients suspected of having a serious infectious disease, such as human immunodeficiency virus, hepatitis B virus (HBV) or HCV; iii) other subjects deemed ineligible by the principal investigator or the investigator in charge of the study. Written informed consent was given by the legal guardians of all study subjects and this study was approved by the Research Ethics Committee of Dokkyo Medical University Hospital [Institutional Review Board (IRB) no. 26096].

Blood specimen collection. BD Vacutainer (Becton Dickinson and Company) serum separation tubes were used to collect blood specimens, which were collected once at the beginning of the study. The tourniquet was removed from the arm as soon as blood flowed to prevent hemoconcentration. Care was taken to perform venipuncture in a manner to minimize any complication. 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). In brief, serum was prepared by allowing blood to clot at 25°C for 1 h, then centrifuged at

1,350 x g for 10 min 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) kit (cat. no. SB-00029; SKIMS-BIO Co.) (13), with results expressed in ng/ml. This ELISA detects human EDN with a minimum detection limit of 6.0 ng/ml and 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. In brief, Nunc MaxiSorp 96-well plates were coated overnight at 4°C with 100 µl of mouse anti-human EDN monoclonal antibody (mAb) (cat. no. KBT-K3231066P) diluted in 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; Merck KGaA), 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 <0.6 ng/ml. Between each subsequent step, plates were washed three 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. Standards and diluted samples (100 µl) were applied to the plates and incubated at room temperature for 1 h. After washing, 100 µl of horseradish-peroxidase-labeled mouse anti-human EDN mAb, included in the ELISA kit, was added to the wells and incubated at room temperature for 1 h. After another wash, the peroxidase substrate tetramethylbenzidine (Sigma-Aldrich; Merck KGaA) was added (100 µl/well) and incubated for 10 min 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 Group). sEDN was determined from a dose-response curve by multiplying the value read from the standard curve by the dilution factor.

IgE measurement. The human IgE ELISA quantitation kit (cat. no. CB-0035; CosmoBio) was used to measure total IgE levels. The sensitivity limit for IgE was 15.6 ng/ml.

The subjects underwent a blood test for specific IgE (sIgE) antibodies using a MAST at enrollment and every six months thereafter at each center. Allergens tested using MAST Immunosystems IV (Hitachi Chemical Co.) were classified as food allergens (wheat, milk, egg white, ovomucoid, peanut, soybean, rice, buckwheat, sesame, tomato, peach, kiwi, banana, tuna, salmon, shrimp, crab, pork, beef and chicken); mite allergens (mason mite and house dust), animal allergens (dog dander and cat dander); and pollen allergens (timothy, *Dactylis*, ragweed, wormwood, cedar, *Cupressaceae*, *Alnus* and white birch). *Candida*, *Alternaria*, *Aspergillus* and latex allergens were excluded from the study. If a subject was positive for one allergen in a group, the subject was considered to be positive for the group.

Statistical analysis. This study was powered with a two-sided test with the significance level set at 0.05. For continuous variables, the number of subjects observed, mean, standard deviation, median, minimum and maximum values were

Table I. Characteristics of the patients (n=86).

Parameter	Value
Gender	
Male	61
Female	25
Age at first visit, months	8.7±3.4 (5.2-11.8)
Body height ^a , cm	69.6±5.3
Body weight ^b , kg	8.4±1.0
Clinical symptoms of pediatric asthma	9 (10.5)
Atopic dermatitis	50 (58.1)
Allergic rhinitis	3 (3.5)
Food allergy	48 (55.8)
Eggs	28
Milk	19
Flour	12
Other	7
Other clinical symptoms	5 (5.8)
Asthma	Mild, 8; moderate, 1
With complications of asthma	1 (1.2)
With history of asthma	57 (66.3)
Family history of allergic diseases	
Sibling	25 (29.1)
Father	42 (48.8)
Mother	47 (54.7)
Pets	23 (26.7)
Passive smoking	15 (17.4)

^aValue determined for 34 patients; ^bvalue determined for 36 patients. Values are expressed as the mean ± SD (range) or n (%).

presented, and the frequency and percentage were presented for categorical data. For normally distributed data, continuous data were tested using the two-samples t-test. If data were not normally distributed, the Wilcoxon rank-sum test (Mann-Whitney U-test) was used. All statistical analyses were performed with IBM SPSS Statistics v.24.0 (IBM Corp.).

Results

Patient characteristics. The study included 123 registered patients, comprising 88 males and 35 females. During the study, a total of 37 subjects dropped out: 23 did not visit the clinic for examination, 12 withdrew consent and 2 deviated from the protocol. Finally, the study analyzed 86 subjects and 37 Controls.

Baseline subject characteristics are displayed in Tables I and II. The most common allergic disease was atopic dermatitis [n=50 (58.1%)], followed by food allergy [n=48 (55.8%)] and asthma [n=9 (10.5%)].

Cumulative sensitization rates to allergens. As indicated in Fig. 1, food allergens were the most common, followed by pollen allergens, environmental allergens (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*), animal allergens and fungal allergens. The cumulative sensitization rate of food allergens reached a plateau at one-and-a-half years of age.

Table II. Treatment details at patient registration (n=86).

Parameter	Value
Oral immunotherapy	2 (2.3)
Antihistamines	21 (24.4)
Asthma medication	4 (4.7)
Inhaled steroid medication	3
Leukotriene antagonist	2
Long-acting β ₂ -stimulant	1
Intal inhalation	2
Atopic dermatitis coating	41 (47.7)
Steroid ointment	41
Protopic ointment	1
Food removal	41 (47.7)
Eggs	30
Milk	21
Flour	11
Soya	1
Other	3
Skincare	46 (53.5)
Moisturizer	4 (4.7)

Values are expressed as n (%). ^aIntal is an anti-inflammatory medication (cromolyn sodium) used in the management of asthma.

Fig. 2 presents the cumulative sensitization rate of each food allergen for all subjects (n=86). Egg white was the most common food allergen, with a cumulative sensitization rate of >80%. By the end of the 3-year study period, food allergens with a cumulative sensitization rate of >50% were as follows: Egg white, ovomucoid, milk, wheat, soya and peanuts. All other food allergens had a cumulative sensitization rate of <50%.

Fig. 3 displays the effects of keeping pets on allergen development. Of note, the group with pets (n=23) was much more likely to develop an animal allergen by the age of 3 years than the group that did not have pets (n=63) (60 vs. 30%). Because of this, animal allergens were the 2nd most common type of allergen in the group with pets. Pollen and environmental allergens were common (>50% cumulative sensitization rate) in both groups.

Cumulative sensitization rates were similar between males (n=61) and females (n=25), with food allergens being the most common, followed by pollen, environmental, animal and fungal allergens (data not shown).

Subjects were divided into those exposed to smoking in the house (i.e., passive smoking) (n=15) and those that were not (n=71). The 'passive smoking group' initially began to develop an environmental allergy at an earlier age, but by the age of 3 years, the 'no passive smoking group' had a much higher cumulative sensitization rate than the passive smoking group (50% vs. 35%) (data not shown).

In Fig. 4, subjects were divided into those who had developed an airway allergic disease, asthma or allergic rhinitis (Onset Group) and those who did not develop any airway allergic disease (Non-onset Group) by the age of 3. The EDN value of each group was measured at 3 time-points: Before 1 year of age, before 2 years of age and before 3 years of age.

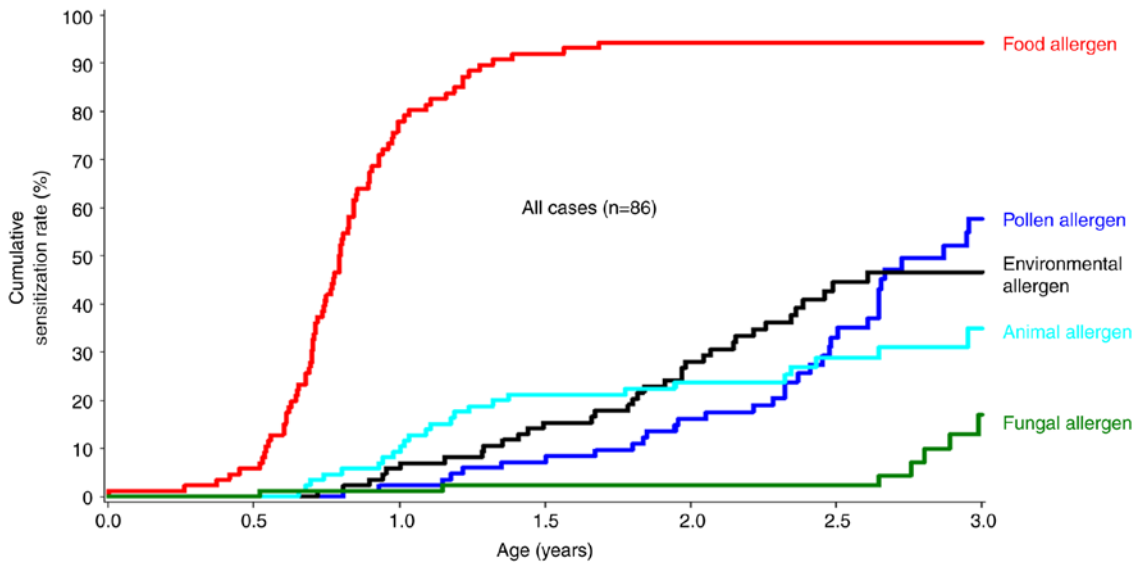


Figure 1. Cumulative sensitization rate of allergens (n=86).

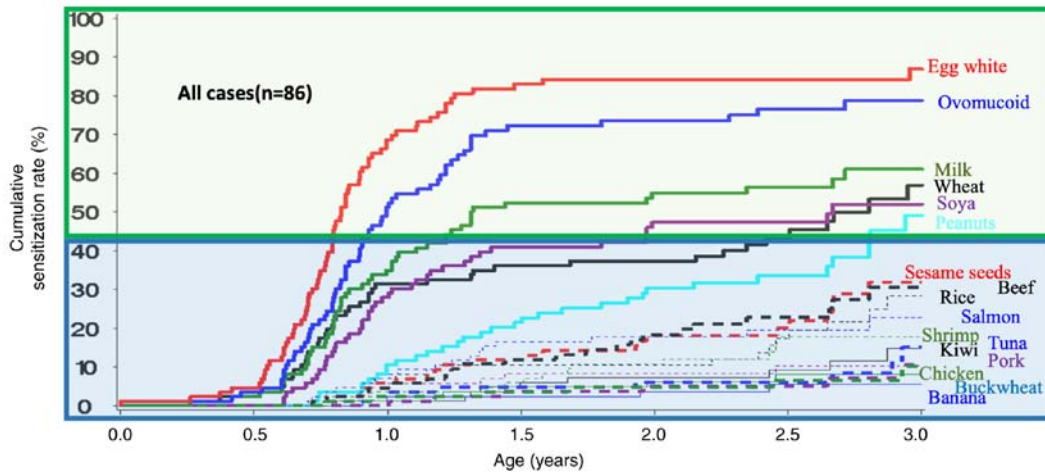


Figure 2. Cumulative sensitization rate of each food allergen (n=86).

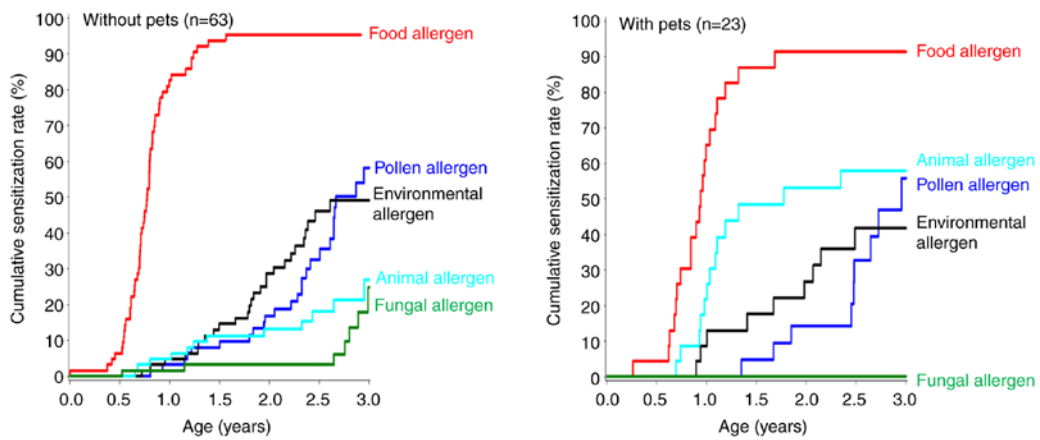


Figure 3. Influence of pets on cumulative sensitization rate. Subjects with pets (n=23) (right) were more likely to develop animal allergens than those without pets (n=63) (left).

Before 1 year of age, the EDN values in each group were similar (84.5 vs. 89.3 ng/ml; Onset Group vs. Non-onset

Group). However, before 2 years of age, the EDN values exhibited a marked divergence (226.6 vs. 65.0 ng/ml; $P < 0.01$)

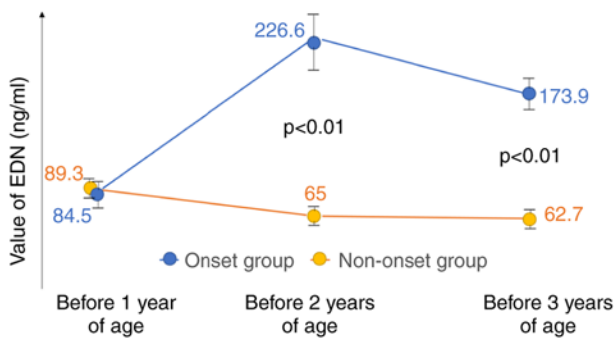


Figure 4. Comparison of EDN levels in Onset Group and Non-onset Group. The Onset Group had significantly higher EDN levels before 2 years of age and before 3 years of age compared to the Non-onset Group ($P<0.01$). Prior to 1 year of age, EDN levels in both groups were statistically similar. EDN, eosinophil-derived neurotoxin.

and remained divergent at the 3rd time-point (before 3 years of age) (173.9 vs. 62.7 ng/ml; $P<0.01$).

In Fig. 5, EDN values before diagnosis were compared between the two groups: Onset and Non-onset. The EDN levels in the Onset Group were much greater than those in the Non-onset Group (171.2 ± 34.28 vs. 81.3 ± 10.02 ng/ml; $P=0.003$). The Onset Group consisted of 4 subjects with asthma and 6 with AR.

Looking at the two groups [Onset ($n=10$) and Non-onset ($n=67$)], EDN levels were compared twice: i) Before 1 and 2 years of age; and ii) before 1 and 3 years of age. There was no significant difference in the first comparison but there was a significant difference in the second ($P=0.001$) (data not shown).

When EDN levels in subjects grouped according to initial symptoms were compared [AD ($n=22$) vs. FA ($n=15$) vs. AD+FA ($n=37$)], there were no significant differences (data not shown).

Discussion

To date, only a small number of studies using EDN levels as a predictor of airway allergic disease in preschool children have been published (6,9,11). In the present study, it was found that young children with elevated EDN levels were more likely to develop allergic disease in their first three years of life. Over the last decades, it has been demonstrated that the presence of one allergic condition may increase the risk for the development of others (1,2,15,16). This 'march' typically begins with AD and then progresses to IgE-mediated FA, asthma and AR. It is therefore highly beneficial to determine factors that may increase the likelihood of allergic disease development. One of these factors may be elevated eosinophil activity.

Allergies may develop at a young age. In the present study, an inclusion criterion was the presence of a food allergen, so all subjects were sensitive to at least one type of food. The other allergen groups (i.e., pollen, environmental, animal and fungal) were much less common, with only pollen allergens exceeding a 50% cumulative sensitization rate by the age of 3 years. There may also be a difference between individuals sensitive to only one food (termed 'monosensitization') and those sensitive to two or more food types (termed 'polysensitization'). A study

of Korean children found that risk factors for polysensitization included parental history of allergic diseases (i.e., AD), birth season (i.e., summer/fall) and exclusive breastfeeding in the first 6 months of life (17). Allergic sensitization may have an important synergistic role in the atopic march. A Canadian longitudinal study investigated whether allergic sensitization enhances associations between atopic dermatitis in infancy with subsequent allergic diseases, including asthma, FA and AD (15). They found that AD without concomitant allergic sensitization was not associated with any increased risk of asthma at 3 years of age, but AD with allergic sensitization increased the asthma risk >7 -fold. They also found that AD and allergic sensitization increased the risk of food allergy development.

In the patients of the present study with household pets, animal allergy was much more likely to develop than in patients without pets. The role of early exposure to pets in the development of atopy remains controversial, with studies producing conflicting results (18-21). The amount of allergen exposure may be a possible explanation for this, as evidence exists that exposure to high levels of pet allergens (i.e., more pets) may decrease a child's risk of sensitization by inducing immune tolerance. Studies have demonstrated an inverse dose-response association between early-life pet exposure and risk of asthma and allergy (22,23).

Before 1 year of age, two groups of the present study (Onset and Non-onset) exhibited statistically similar EDN levels. However, as the patients aged, EDN levels exhibited a marked divergence before 2 years of age and maintained this difference up until 3 years of age, which was the endpoint of the present study. Elevated EDN levels appear to be linked to onset of airway allergic disease. It should also be noted that children exhibited increased EDN levels before the onset of airway allergic disease. Elevated EDN levels are a sign of eosinophil activity (i.e., activation and degranulation).

Typically, patients under the age of 6 years cannot fully participate in traditional measures of lung function, therefore making airway disease diagnosis difficult. A delay between elevation of immune cells, e.g. eosinophils, and onset of lung dysfunction is frequently observed (24). If treatment decisions are based solely on lung function, this may lead to a delay in care and unnecessary morbidity in the patient. Biomarkers are measurable indicators that link an underlying pathophysiological pathway to a disease (25,26). Finding a reliable and accurate biomarker for inflammatory disease, such as asthma, AD and FA, may be an invaluable tool for diagnosis, treatment and monitoring. One such possible tool is EDN, an eosinophil granule protein released almost exclusively by eosinophils (4). Therefore, EDN levels would directly correspond with eosinophil activity. Several studies have demonstrated the efficacy of EDN levels as a biomarker for eosinophilic inflammation (9,6-11,27-30), highlighting its ability to represent the secretory activity of eosinophils, a combination of the concentration of eosinophils and their tendency to release degranulation products. Measuring EDN is quick, simple and accurate with the recently developed K-EDN ELISA kit. EDN is more easily recovered from serum specimens than other eosinophil granule proteins, such as ECP, and may be recovered from numerous different specimen types, including sputum,

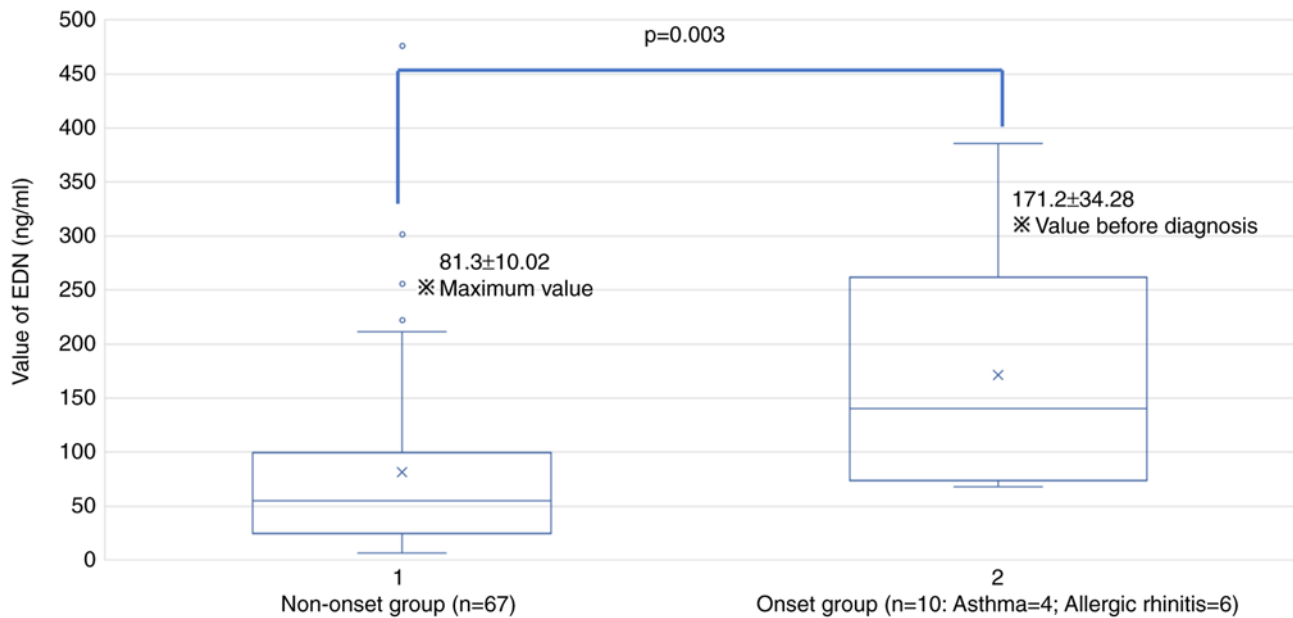


Figure 5. Comparison of maximum EDN levels between Onset Group and Non-onset Group. EDN levels prior to allergic disease diagnosis in the Onset Group were significantly higher than those in the Non-onset Group ($P=0.003$). EDN, eosinophil-derived neurotoxin.

saliva, nasal and bronchoalveolar lavage, serum, plasma and urine (5,29).

Putting into practice EDN as a diagnostic tool has led to promising results. A study by Kim *et al* (8) found that EDN levels had predictive value for asthma [sensitivity, 81.3%; specificity, 87.1%; positive predictive value (PPV), 90.7%; negative predictive value (NPV), 75.0%]. A more recent study by Amer *et al* (31) also demonstrated the predictive value of EDN for asthma [sensitivity, 100%; specificity, 64.7%; PPV, 91.9%; NPV, 100%] and a strong correlation with severity. Among the patients of the present study, 10 eventually developed allergic disease (4 with asthma and 6 with allergic rhinitis). In this group, EDN levels were significantly higher at 'prior to 2 years of age' and 'prior to 3 years of age' when compared to the group with no onset of allergic disease. EDN has also been used as a predictive biomarker for recurrent wheezing development after RSV bronchiolitis, a common airway infection in young children. Using 53 ng/ml as a cutoff value, EDN levels at 3 months after RSV infection had a PPV of 57%, NPV of 76%, sensitivity of 72% and specificity of 62% for recurrent wheezing (11).

The present study highlights the utility of EDN as a biomarker for allergic disease development, helping clinicians diagnose, treat and monitor underlying eosinophilic inflammation. EDN levels allow the clinician to stratify patients according to treatable eosinophilic inflammation (32) and have the advantage of being measurable in several biological fluids, even in young children. More studies are needed looking at the potential link between elevated EDN levels and airway allergic disease in young children.

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Availability of data and materials

The data used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

YT, CKK and SY designed the study. YT, CKK and SY checked and confirmed the authenticity of the raw data. YT, CKK, ZC, JP, ShinY, MK and ShigY collected, analyzed and interpreted

the data. YT, CKK, ZC and SY wrote the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All legal guardians of patients provided written informed consent before participating in this study and this study was approved by the Research Ethics Committee of Dokkyo Medical University Hospital (approval no. 26096).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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