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ORIGINAL ARTICLE **Asthma and Rhinitis**

Relationship between sputum clusterin levels and childhood asthma

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Summary

Background Clusterin is a sensitive cellular biosensor of oxidative stress and has been studied as a biomarker for inflammation-associated diseases. Clusterin levels in childhood asthma have not been evaluated.

Objectives (1) To evaluate sputum clusterin levels in children with asthma compared to a control group. (2) To assess the relationships between sputum clusterin levels and airway inflammation, pulmonary function, and bronchial hyperresponsiveness.

Methods This study included 170 children aged 5–18 years with stable asthma (n = 91), asthma exacerbation (n = 29), or no asthma (healthy controls; n = 50). Induced sputum, pulmonary function, and methacholine challenge tests were performed. Stable asthma was classified into two groups according to the severity. Clusterin levels in sputum were measured using an enzyme-linked immunosorbent assay.

Results Children with stable asthma had a higher clusterin level than healthy controls [4540 (3872–5651) pg/mL vs. 3857 (1054–4369) pg/mL, $P < 0.001$]. The clusterin level was also more elevated in eosinophil-dominant sputum than in non-eosinophilic sputum in stable asthma [5094 (4243–6257) pg/mL vs. 4110 (1871–4839) pg/mL, $P = 0.0017$]. Clusterin levels were associated with asthma severity. Paradoxically, clusterin levels were lower during asthma exacerbation than in stable asthma [1838 (350–4790] pg/mL vs. 4540 (3872–5651) pg/mL, $P < 0.001$]. Clusterin levels were strongly correlated with the methacholine concentration that caused a 20% decrease in the forced expiratory volume in 1 s ($r = -0.617$, $P < 0.001$); there was no significant correlation between clusterin levels and other pulmonary function parameters.

Conclusions and Clinical Relevance Clusterin levels were altered in children with stable asthma and asthma exacerbation because of its antioxidant and anti-inflammatory effects. Clusterin may be a marker that reflects airway inflammation and severity of symptoms, and it can be used in the assessment and management of childhood asthma.

Keywords airway inflammation, asthma exacerbation, clusterin, stable asthma
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Introduction

Asthma is a chronic airway inflammatory disorder associated with variable airflow obstruction and bronchial hyperresponsiveness (BHR) and presents with recurrent episodes of wheezing, coughing, shortness of breath, and chest tightness [1]. There has been increasing interest and trials in the non-invasive assessment of airway inflammation, clinical asthma control, and the effect of treatment. Conventional tests such as bronchodilator response (BDR) or provocation tests for BHR are only indirectly associated with airway inflammation [2]. Measurement of fractional nitric oxide (FeNO) levels in exhaled breath has been recommended as a quantitative, non-invasive, and safe method for the diagnosis of eosinophilic airway inflammation [2]. However, its applicability in children is limited owing to a lower measurement success rate and a relatively poor accuracy and detection limit at low FeNO levels [3].

Clusterin is a heterodimeric glycoprotein in which α and β chains are interconnected via five disulphide bonds, is comprised of 449 amino acids, and has a molecular weight of approximately 80 kDa [4–6]. It is present in most human organs, in numerous physiologic

fluids, including semen, urine, breastmilk, cerebrospinal fluid, and plasma; and in specific cell types in almost all types of mammalian tissue [7, 8]. For example, it is expressed in epithelial cells at tissue–fluid interfaces, specific subpopulations of neurons, and pancreatic acinar cells, and accumulates in the artery wall during the development of atherosclerosis [9, 10]. Extracellular clusterin synthesis is increased following disturbances such as hypoxia, stress, cell death, or injury, and it functions as a multi-functional cell protector that sequesters harmful agents during tissue remodelling and degeneration and helps with their removal [11]. Clusterin has been functionally implicated in several physiological processes, including many pathological conditions such as ageing, heat shock, UV radiation, oxidative stress, diabetes, atherosclerosis, degenerative diseases, renal disease, and tumours [6, 8]. More specifically, it protects normal human fibroblasts from cytotoxicity induced by UVB, human prostate cancer cells from oxidative stress-induced DNA damage, lung fibroblasts from cigarette smoke oxidants, and human retinal cells from free radical damage [6]. Clusterin also has anti-apoptotic and anti-inflammatory functions [10], has a protective role in pancreatitis, enhances the clearance of amyloid-β from the brain by accelerating transport across the blood–brain barrier, and prevents excessive inflammation in Alzheimer's disease by inhibiting the complement system and NF-κB [12].

A few studies have investigated the relationship between clusterin levels and oxidative stress or inflammatory disease [10, 12, 13]. Although there have been many studies concerning the relationship between specific diseases and clusterin expression in associated tissues or body fluids; elevated clusterin in atherosclerosis, pancreatitis, or type 2 diabetes [14, 15]; and suppression of clusterin in nephrotic syndrome or chemotherapeutic sensitive human cancer [16, 17], the relationship with asthma has not yet been well studied. Kwon et al. [13] was the first to demonstrate a relationship between clusterin and oxidative stress in asthmatic patients; the latter is a core feature in the pathogenesis of asthma [18]. Therefore, clusterin may be involved in airway inflammation.

The aim of this study was to assess the relationship between sputum clusterin levels and childhood asthma and to investigate the correlation of sputum clusterin levels with airway inflammation, pulmonary function, and BHR.

Materials and methods

Study design

We enrolled 211 children who visited the Severance Children's Hospital for work-up or treatment of asthma or routine health check-up between June 2012 and September 2014.

Asthma was diagnosed on the basis of current episodic respiratory symptoms such as recurrent cough or dyspnoea, shortness of breath, chest tightness, and physician's diagnosis of asthma and airway hyperresponsiveness according to the guidelines of the American Thoracic Society [19].

Stable asthma was defined as no asthmatic exacerbation during the preceding 4 weeks the necessitated systemic corticosteroids or an increased use of inhaled corticosteroids, the use of rescue treatment ≤ 3 times a week, and no clinical indication for a change in medication; asthma exacerbation was defined as a worsening of asthma requiring the use of systemic corticosteroids or hospitalization to prevent a serious outcome [20–22]. Healthy controls had normal lung function without airway hyperresponsiveness and had never had a doctor's diagnosis of asthma. Children with stable asthma were also classified as having intermittent-to-mild persistent asthma or moderate-to-severe persistent asthma based on the guidelines from the National Heart, Lung, and Blood Institute [23].

Spirometry was performed, and sputum samples were obtained at the initial visit for evaluation of eosinophil levels. Sputum was induced within 24 h after presentation; children who underwent sputum induction after 24 h because of insufficient post-BD FEV_1 were excluded.

A methacholine challenge test was performed, and at the second visit, total serum IgE and specific IgE levels were measured using the Pharmacia CAP assay (Uppsala, Sweden). A specific IgE test was performed with six common allergens in Korea, including *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, egg white, cow's milk, German cockroach, and *Alternaria alternata*. Atopy was defined as ≥ 0.7 KUa/L specific IgE to more than one allergen or 150 IU/mL total IgE.

This study was approved by the Institutional Review Board of Severance Hospital (protocol no. 4-2004-0036). Written informed consent was obtained from the participants and their parents.

Sputum induction and processing

Sputum induction and processing were performed as previously described by Yoshikawa et al. [24, 25]. Sputum induction was previously reported successful, defined as obtaining a readable sputum cytospin, in 68–100% of children [26]. All children were instructed to wash their mouths thoroughly with water, after which they inhaled a 3% saline solution nebulized in an ultrasonic nebulizer (NE-U12; Omron Co., Tokyo, Japan) at maximum output at room

temperature. The children were encouraged to cough deeply at 3-min intervals thereafter. After sputum induction, spirometry was repeated. If the FEV_1 was ≤ 80% of the post-BD baseline value, the child was required to wait until it returned to baseline values [27]. Sputum samples were kept at 4°C for no more than 2 h before further processing. A portion of the samples was diluted with a phosphate-buffered saline (PBS) solution containing 10 mmol/L of dithiothreitol (WAKO Pure Chemical Industries Ltd, Osaka, Japan) for cell count and gently vortexed at room temperature for 20 min. After centrifugation at 400 g for 10 min, the cell pellet was resuspended in PBS. We used trypan blue exclusion to ensure adequate viability. Total cell counts were performed using a haemocytometer, and slides were prepared with cytospin (Cytospin3; Shandon, Tokyo, Japan) and stained with May–Grünwald–Giemsa stain for differential cell counts. Airway eosinophilia was defined as an eosinophil count ≥ 3% of the total cell count [22, 28, 29]. The supernatant was then stored at −70°C for the subsequent assay of clusterin levels.

Spirometry and methacholine challenge test

Spirometry (VIASYS Healthcare, Inc., Conshohocken, PA, USA) was performed, and flow–volume curves were obtained according to the American Thoracic Society guidelines before and after BD inhalation [30]. BHR was assessed using the methacholine challenge test according to standardized procedures [31]. Each child inhaled increasing concentrations of methacholine (0.075, 0.15, 0.31, 0.62, 1.25, 2.5, 5, 10, 25 and 50 mg/mL) nebulized by a dosimeter (MB3; Mefar, Brescia, Italy) until FEV_1 decreased 20% from a post-nebulized saline solution value. The concentration of methacholine that caused a 20% decrease in FEV_1 (PC_{20}) was determined, and the result for a challenge was considered positive if the PC_{20} was ≤ 16 mg/mL.

Measurement of clusterin levels from induced sputum

Sputum clusterin levels were measured using a commercially available enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Statistical analyses

Children were classified into three groups [control, stable asthma (intermittent-to-mild persistent asthma and moderate-to-severe persistent asthma), and asthma exacerbation], and comparisons were conducted between two of three groups: control vs. stable asthma and stable asthma vs. asthma exacerbation.

Normally distributed data were analysed using the Student's *t*-test; non-normally distributed data were analysed using the Mann–Whitney *U*-test. Comparisons among the three groups of control, intermittent-to-mild persistent asthma, and moderate-to-severe persistent asthma were performed with the Kruskal–Wallis test, and multiple comparisons were conducted using Dunn's method. Correlations between clusterin levels and pulmonary function parameters, PC_{20}, and sputum eosinophil percentage were evaluated using the Spearman test.

All statistical analyses were performed using SPSS 20 (IBM Corp, Armonk, NY, USA). A result was deemed significant at $P < 0.05$.

Results

Characteristics of subjects with stable asthma and healthy controls

Of the 211 children enrolled, we evaluated results for 170 children ($n = 91$, stable asthma; $n = 29$, asthma exacerbation; $n = 50$, healthy controls; Appendix S1). The median age was 9.3 years, and the male to female ratio was 1.9 : 1.

Blood eosinophil counts were not different between stable asthma and healthy controls. The proportion of eosinophils in sputum was much higher in the patients with stable asthma than in the healthy control group [2% (0–10%) vs. 0% (0–1%), $P < 0.001$]. Compared with the healthy controls, the patients with stable asthma also had a lower percentage of FEV_1, FEV_1/forced vital capacity (FVC), and forced expiratory flow at 25–75% of vital capacity ($FEF_{25–75\%}$), indicating airway obstruction, and a greater change in FEV_1 after BD inhalation. They had a much greater tendency for atopy than the healthy controls [69/87 (79.3%) vs. 26/45 (57.8%), $P = 0.009$; Table 1]. Of those with stable asthma, patients with moderate-to-severe persistent asthma had a lower percentage of predicted FEV_1, FEV_1/FVC, and $FEF_{25–75\%}$, and a higher BDR than the patients with intermittent-to-mild persistent asthma. The sputum eosinophil percentage was higher in moderate-to-severe persistent asthma than in intermittent-to-mild persistent asthma, but there was no difference in the tendency for atopy (Table 1).

Clusterin levels in stable asthma and healthy controls

Clusterin levels in the sputum supernatant were higher in those with stable asthma than in healthy controls [4540 (3872–5651) pg/mL vs. 3857 (1054–4369) pg/mL, $P < 0.0001$; Fig. 1].

Clusterin levels were higher in patients with moderate-to-severe persistent asthma than in those with intermittent-to-mild persistent asthma [4903 (4503–6190) pg/mL vs. 4262 (2799–5172) pg/mL, $P < 0.01$; Fig. 1].

Table 1. Characteristics of the study population

Characteristics	Controls (n = 50)	Stable asthma (n = 91)		
		Intermittent-to-mild persistent asthma (n = 63)	Moderate-to-severe persistent asthma (n = 28)	Asthma exacerbation (n = 29)
Age, years	9.8 (8.7–12.0)†	8.1 (6.5–10.5)	10.9 (7.4–12.9)	8.5 (6.3–12.6)
Sex, male (%)	33 (66.0)	41 (65.1)	23 (82.1)	15 (51.7)
Blood eosinophil, /μL	310 (150–445)	365 (208–655)	390 (150–650)	195 (32.5–670)
Sputum eosinophil, %	0 (0–1)†	2 (0–5)	4 (0.25–16)§	0 (0–17)
Sputum neutrophil, %	65 (39–84)	55 (34–81)	32 (16–57)	53 (34–79)
Total IgE, IU/mL (n = 127)	148 (69–443)	259 (100–598)	261 (98–684)	299 (190–677)
Atopy, n (%) (n = 132)	26/45 (57.8)	49/60 (81.7)	20/27 (74.1)	21/25 (84.0)
ICS-treatment, n (%)	NA	11 (17.5)	5 (17.9)	5 (17.2)
ICS dose* (μg)	NA	216 ± 94	223 ± 114	284 ± 143¶
Systemic steroid use, n (%)	NA	NA	NA	26/29 (90.0)
FEV_1, % predicted	110.7 ± 13.1†	105.9 ± 14.4‡	88.7 ± 18.7§	79.4 ± 20.4¶
Change in FEV_1 after BD, %	2.3 ± 3.5†	6.0 ± 6.2‡	10.1 ± 8.6§	6.1 (1.65–19.4)
FEV_1/FVC, %	89.1 ± 4.7	90.2 ± 6.5‡	79.8 ± 13.8§	78.5 ± 12.4¶
$FEF_{25–75\%}$, % predicted	105.5 ± 18.3†	93.1 ± 23.2‡	62.5 ± 31.2§	56.5 ± 31.9¶

Values are presented as mean ± standard deviation, median (interquartile range) or number (%).

ICS, inhaled corticosteroids; FEV_1, forced expiration volume in one second; BD, bronchodilator; FVC, forced vital capacity; $FEF_{25–75\%}$, forced expiratory flow at 25–75% of vital capacity; NA, not applicable.

*Budesonide equivalent dose.

$P < 0.01$, as compared with †intermittent-to-mild persistent asthma, ‡moderate-to-severe persistent asthma, or §healthy controls using the ANOVA or Kruskal–Wallis test and Bonferroni or Dunn's method for multiple comparisons.

¶$P < 0.01$, as compared with stable asthma using the Mann–Whitney U-test.

Clusterin levels and airway inflammation in stable asthma

Clusterin levels were higher in patients with eosinophilic airway inflammation (induced sputum eosinophils ≥ 3%) than in those with non-eosinophilic stable asthma [5094 (4243–6257) pg/mL vs. 4110 (1871–4839) pg/mL, P = 0.0017; Fig. 2a]. Moreover, clusterin levels were weakly correlated with the percentage of sputum eosinophils (r = 0.250, P = 0.017; Fig. 2b).

Clusterin levels and other parameters related with stable asthma

Approximately 40% (36/91) of patients with stable asthma showed positive results in the methacholine

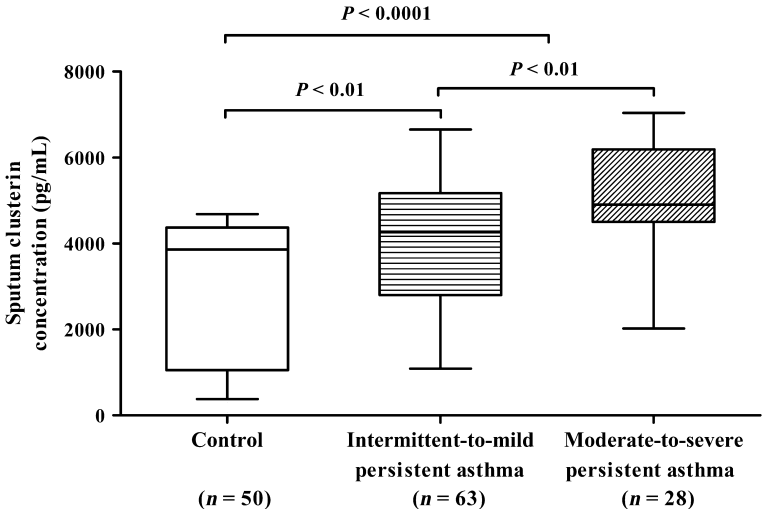

Fig. 1. Clusterin levels in supernatants of induced sputum from healthy control subjects and patients with stable asthma (intermittent-to-mild persistent and moderate-to-severe persistent asthma). The Kruskal–Wallis test was conducted for comparisons among three groups ($P < 0.001$), followed by multiple comparisons using Dunn's method ($P < 0.01$). Clusterin levels were significantly higher in patients with stable asthma than in healthy control subjects. Clusterin levels were lower in intermittent-to-mild persistent asthma than in moderate-to-severe persistent asthma. Box-and-whisker plots represent the 25th and 75th percentiles, with the median line and error bars representing the 10th and 90th percentiles.

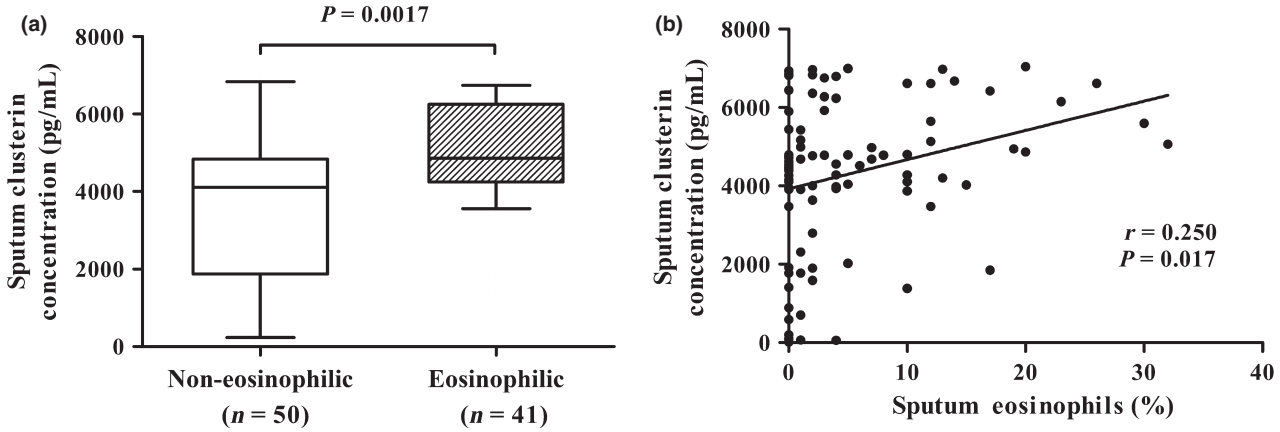

Fig. 2. Association between sputum clusterin levels and eosinophilic airway inflammation. (a) Clusterin levels of eosinophilic sputum were higher than that of non-eosinophilic sputum in stable asthma. Box-and-whisker plots represent the 25th and 75th percentiles, with the median line and error bars representing the 10th and 90th percentiles. (b) Sputum clusterin levels showed a significant correlation with the percentage of sputum eosinophils ($r = 0.250$, $P = 0.017$).

challenge ($PC_{20} \leq 16$ mg/mL). Clusterin levels had a strong inverse correlation with PC_{20} in those with BHR ($r = -0.617$, $P < 0.001$; Fig. 3); it was not correlated with FEV_1, $FEF_{25-75\%}$, or BDR in patients with stable asthma. In addition, clusterin levels were not different between atopy and non-atopy in those with stable asthma or healthy controls (data not shown).

Clusterin levels in asthma exacerbation

The stable asthma and asthma exacerbation groups had no significant difference in age, sex, blood and sputum eosinophil counts, total IgE, atopy tendency, or BDR. The percentage of predicted FEV_1, FEV_1/FVC, and $FEF_{25-75\%}$ were lower during asthma exacerbation than in stable asthma (Table 1).

Unexpectedly, clusterin levels during asthma exacerbation were lower than during stable asthma [1838 (350–4790) pg/mL vs. 4540 (3872–5651) pg/mL, $P < 0.001$; Fig. 4].

Discussion

This study showed that sputum clusterin levels were higher in patients with stable asthma than in healthy controls; furthermore, the sputum clusterin levels increased with asthma severity for patients with stable asthma. We also found that sputum clusterin levels were associated with eosinophilic airway inflammation and BHR in stable asthma. However, clusterin levels

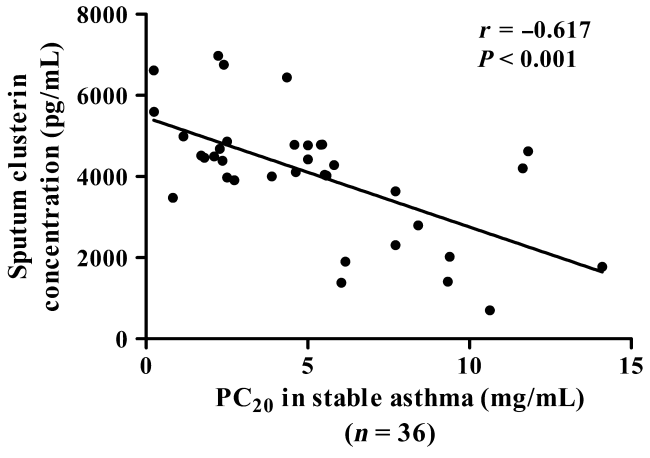

Fig. 3. Association between sputum clusterin levels and methacholine concentration causing a 20% decrease in forced expiratory volume in one second (PC_{20}) in stable asthma. Sputum clusterin showed a strong, significant negative correlation with PC_{20} in stable asthma ($r = -0.617$, $P < 0.001$).

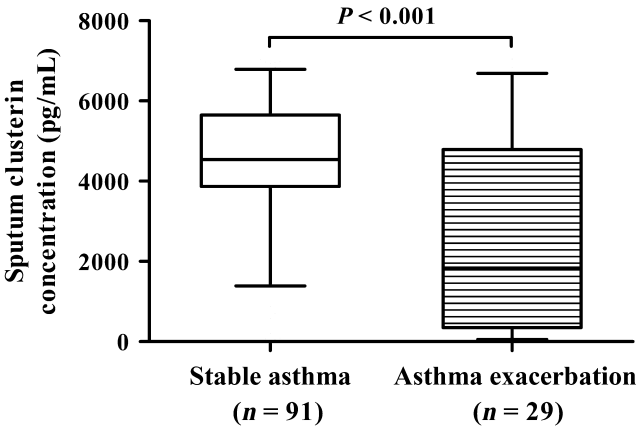

Fig. 4. Sputum clusterin levels from children with asthma exacerbation. Clusterin levels during asthma exacerbation were significantly lower than those in stable asthma.

were lower for patients with asthma exacerbation compared with both patients with stable asthma and healthy controls.

Sputum clusterin levels were higher in children with asthma than in the healthy controls, which supports a previous study that demonstrated elevated clusterin levels in serum and sputum from adults with asthma [13]. The elevated clusterin in childhood asthma might also be attributed to oxidative stress, which plays an important role in asthma pathogenesis.

Sputum clusterin levels in children were also higher with greater asthma severity, as based on clinical symptoms, similar to an earlier study of adult asthma [13]. There is strong evidence that endogenous and exogenous reactive oxygen and nitrogen species play a major role in airway inflammation and are determinants of asthma severity [32], and the presence of strong oxidative stress in children with asthma also increases with the severity of the disease [33]. Clusterin is a sensitive cellular biosensor of oxidative stress that functions to protect cells from the deleterious effects of free radicals and their derivatives, and it suppressed the deleterious effects of oxidants in several studies [6]. Based on this, the increase in clusterin levels with asthma severity is likely related with the oxidative stress in patients with asthma.

Induced sputum is the most common method for defining inflammatory phenotypes; moreover, different inflammatory cell profiles in sputum from patients with asthma can be identified, and these may predict response to treatment [34]. Accordingly, sputum eosinophil percentages are a strong predictor of airway inflammation [35]. In the present study, sputum clusterin was higher in eosinophil-dominant airway inflammation than in non-eosinophilic airway inflammation; furthermore, sputum clusterin showed a weak positive correlation with eosinophilic airway inflammation. This indicates that increased clusterin with eosinophilic airway inflammation is a characteristic of childhood asthma. However, sputum clusterin and atopy were not related, despite the weak, but significant, association with airway eosinophils, and atopy and blood eosinophil count did not differ among the groups despite the significant difference in the eosinophil portions of the sputum. The might be attributed to the independence of atopy and eosinophilia based on asthma phenotype [36], and it is possible that sputum clusterin levels reflect selective airway eosinophilia in asthma.

In the present study, sputum clusterin levels in asthmatic children were not related with FEV_1 or $FEF_{25–75\%}$ values, which represent bronchial obstruction; however, they inversely correlated with PC_{20}, which represents airway hyperresponsiveness. A number of studies have suggested that predicted FEV_1 is poorly correlated with asthma severity, as classified based on symptom frequency or medication usage, and the degree of airway responsiveness is linked to disease severity in children with mild-to-moderate asthma [37–39]. The present study showed that PC_{20} is inversely correlated with clusterin levels; therefore, airway inflammation and oxidative stress might contribute to the development of BHR [40, 41].

In contrast, clusterin levels during asthma exacerbation were lower than those in children with stable asthma or in healthy controls. In a previous study in which serum clusterin was measured before and after initial inhaled corticosteroid treatment in patients with asthma, the levels were lower after treatment than before [13]. In the same context, clusterin levels might also have been lower following corticosteroid treatment in the present study because sputum was induced after at least one administration of systemic corticosteroids for most of the children with asthma exacerbation who required systemic corticosteroids. Clusterin reduces oxidative stress and prevents excessive inflammation [12]. Studies have proposed that serum clusterin in vascular diseases, such as coronary heart disease and myocardial infarction, increases with disease-related stress as an indicator of vascular damage; in contrast, accumulated clusterin functions to protect against disease-related oxidative stress [9, 42]. Based on these dual functions of clusterin, it could be also assumed that lower clusterin levels during asthma exacerbation are likely associated with an imbalance between the antioxidant function of clusterin and the excessive oxidative stress in asthma exacerbation. Lastly, relatively lower levels of clusterin were observed in high-density lipoprotein during acute influenza infection in a study of atherosclerosis, suggesting that the influenza virus infection attenuated clusterin expression [43, 44]. Therefore, clusterin levels could also decrease after a viral respiratory infection that causes asthma exacerbation in children.

The present study has a few limitations. First, the study did not investigate FeNO levels. Consequently, the usefulness of clusterin as an airway inflammatory marker could not be compared to that of FeNO, which serves as one of the representative markers of eosinophilic airway inflammation. Second, systemic steroid administration was not controlled for in patients with asthma exacerbation. Accordingly, the relationship between clusterin levels and asthma exacerbation without the effect of steroid could not be determined. Nevertheless, to our knowledge, this is the first study showing elevated clusterin levels in childhood asthma and suggesting that clusterin may decrease during asthma exacerbation.

In conclusion, sputum clusterin levels were higher in stable asthma, increased with asthma severity, but

dropped to levels lower than that observed in stable asthma or healthy controls during asthma exacerbation. Clusterin levels were also correlated with eosinophilic airway inflammation and BHR. These findings suggest that sputum clusterin may be a surrogate biomarker for the assessment and management of asthma in children with asthma. Increased clusterin levels with greater asthma severity may be a protective response against the development of oxidative stress and airway inflammation in asthma. The functional relationship between lower clusterin levels and asthma exacerbation needs to be studied further using a clusterin knockout asthma mouse model.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Selection of stable asthma subjects.
Appendix S2. Serum clusterin levels for patients with stable asthma and healthy controls.