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Original Article

Determination of the cutoff values of Th2 markers for the prediction of future exacerbation in severe asthma: An analysis from the Hokkaido Severe Asthma Cohort Study

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ACT, asthma control test; ANOVA, analysis of variance; AQLQ, asthma quality of life questionnaire; ATS, American Thoracic Society; CFE, consistent frequent exacerbators; CI, confidence interval; CNE, consistent non-exacerbators; ED, emergency department; Eo, blood eosinophil; ERS, European Respiratory Society; FeNO, fraction of exhaled nitric oxide; HR, hazard ratio; IE, intermittent exacerbators; OCS, oral corticosteroid; OR, odds ratio; ROC, receiver operating characteristic; Th2, T helper 2

ABSTRACT

Background: We recently reported that severe asthma patients with frequent exacerbations showed high blood eosinophil counts (Eo) and fractions of exhaled nitric oxide (FeNO) compared with non-exacerbators. However, we did not determine exact cutoff values related to exacerbation. The aim of this study was to determine the cutoff values of Eo and FeNO that could be related to the exacerbation of severe asthma. We also aimed to confirm the clinical utility of Th2 markers related to exacerbation.

Methods: This study included 105 severe asthma patients who completed a three-year follow-up of a severe asthma cohort study, including smokers. Three Th2 markers were selected, viz., Eo, FeNO, and positive atopic status. Regarding Eo and FeNO, we determined the cutoff values for the definition of "positive" Th2 features using receiver operating characteristic curve analyses, based on the comparisons between frequent exacerbators and other patients.

Results: The cutoff values for positive Th2 features were Eo ≥ 250 cells/ μ L and FeNO ≥ 31 ppb. Sixteen patients (15.2%) had no Th2 features, 40 (38.1%) had one, 25 (23.8%) had two, and 24 (22.9%) had three. A high number of positive Th2 features was significantly associated with exacerbation frequencies over three years ($p < 0.05$). Similarly, compared to patients with one or no Th2 features, those with three Th2 features had a significantly higher probability of having more than one exacerbation ($p < 0.05$).

Conclusions: The cutoff values determined in the current analysis were good predictors of future exacerbations in severe asthma patients.

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Introduction

Asthma is recognized as a heterogeneous and complex disease that has distinct therapeutic approaches.^{1–4} Treatment approaches

depend upon its phenotypic classification. Exacerbation-prone asthma has been identified as a potentially distinct phenotype and is linked with high morbidity and mortality.^{5–9} In fact, prevention of asthma exacerbations is one of the major goals of the current international guidelines for asthma treatment.¹⁰ Therefore, to prevent future exacerbations and ultimately reduce health care costs, it is imperative to identify high-risk patients to achieve these goals.

Several retrospective studies have investigated the significance of clinical predictive factors for future asthma exacerbation. These

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factors are the T-helper 2 (Th2)-related inflammatory biomarkers, and include the blood/sputum eosinophil level, immunoglobulin E (IgE) level, and fractional excretion of nitric oxide (FeNO) concentration.^{5,6,11,12} We recently reported that compared to non-exacerbators, patients with frequent exacerbations showed higher Th2 markers.¹³ In this analysis, we categorized all severe asthma patients into three groups based on their exacerbation status during a three-year follow up period. The first group comprised patients that were consistent frequent exacerbators (CFE); the second group comprised patients that were consistent non-exacerbators (CNE), while the third comprised patients that were intermittent exacerbators (IE). Comparison of several Th2 markers among these three groups demonstrated higher blood eosinophil counts (Eo) and FeNO in the CFE group than in the other two groups.¹³

However, the clinical utility of these data is limited, considering that it is only based on a comparison of continuous values among three groups. Therefore, we thought that the exact cutoff value for each exacerbation-related Th2 index needed to be determined. Further, we thought that the combined use of several biomarkers, rather than a single one, would be even more accurate.

In this study, using the same data obtained through the patients' inclusion in the Hokkaido Severe Asthma Cohort Study,¹⁴ we first attempted to determine the exact cutoff levels for Eo and FeNO using receiver operating characteristic (ROC) curve analysis. We aimed to determine the potential utility of three Th2 biomarkers (Eo, FeNO, and atopic status), which can be easily measured in clinics worldwide, for the prediction of future asthma exacerbations.

Methods

The details of the methods used in this study have been described in our previous report¹⁴ and can be found in [Supplementary Methods](#).

Study protocol and patients

The Hokkaido Severe Asthma Cohort Study or the Hokkaido-based Investigative Cohort Analysis for Refractory Asthma (UMIN ID: 000003254) was a multicenter observational cohort study, which primarily aimed to characterize severe asthma patients, including smokers. The details of this study protocol have been reported previously.¹⁴ Severe asthma patients were enrolled at the Hokkaido University Hospital and its 29 affiliated hospitals and clinics between February 2010 and September 2012. Severe asthma was diagnosed on the basis of the American Thoracic Society (ATS) criteria for refractory asthma, published in 2000¹⁵, with slight modifications. Briefly, we included the following additional criteria: 1) a severe asthma diagnosis required fulfilment of one or both major criteria and two minor criteria, and 2) patients whose condition was well under control using medications were asked if they experienced episodic deterioration of symptoms, required urgent care visits, or rescue use of short-acting bronchodilators when the dose of the current medication was reduced within the last year.¹⁴ Overall, 127 severe asthma patients were selected for the baseline analysis. A flow chart demonstrating the study process from the initial screening at entry (VE) to the first- and second-year visits (V1 and V2, respectively), and finally at the end of the three-year follow-up period (V3) is depicted in a previous report.¹³

Assessment of exacerbation

We defined asthma exacerbation based on the need for systemic corticosteroids for more than three days and/or hospital admission.

This corresponds with the severe exacerbation classification by the European Respiratory Society (ERS)/ATS guidelines on severe asthma.¹⁶ Frequent exacerbators were defined as those who experienced two or more exacerbations within one year of follow-up, whereas non-exacerbators were defined as those who did not experience any exacerbations.¹³

Selection and measurement of Th2 markers

In our previous study,¹³ we showed that Eo and FeNO, but not total IgE and periostin were useful biomarkers for predicting future asthma exacerbation episodes. Therefore, in this present study, Eo and FeNO were selected as continuous variables. The categorical variable of atopic status was defined as a positive specific IgE test result for at least one common inhaled allergen. All of these were clinically available. Using the ROC curve, we determined the cutoff values for the definition of positive Th2 features, based on the comparisons between consistent frequent exacerbators and the other patients (intermittent exacerbators and non-exacerbators). FeNO concentration was measured with the NIOX MINO® (Aerocrine, Stockholm, Sweden) using a single-breath online method, in accordance with the ATS guidelines.¹⁷ Details of the methods of measurements of other biomarkers are described in our previous report¹⁴ and can be found in [Supplementary Methods](#).

Statistical analysis

We selected three Th2 markers, which were available for use in the clinics in Japan. These included the following markers:

Eo

FeNO

Atopy [defined as a positive specific IgE for at least one common inhaled allergen; multiple antigen simultaneous test (MAST) scores (>1.01 lumicount) to at least one common inhaled allergen]

An optimal cutoff value was obtained from the highest sum of sensitivity and specificity. To compare the differences among groups, we used the Student's t-test or one-way analysis of variance for continuous parametric variables (ANOVA), Mann–Whitney U-test for continuous nonparametric variables, and chi-square test for categorical variables. To analyze the tendency of continuous and categorical variables among the three exacerbation groups, we performed the Jonckheere–Terpstra trend test. Logistic regression analyses were performed to calculate adjusted odds ratios (ORs) and 95% confidence intervals (CIs). We calculated the hazard ratio (HR) and 95%CI, using the Cox proportional hazards model.

Statistical analyses were performed using the statistical software package SYSTAT for Windows, version 13.1 (SYSTAT, San Jose, CA, U.S.A.) and EZR, version 1.35 (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for the R software (The R Foundation for Statistical Computing, Vienna, Austria).¹⁸ For all statistical analyses, $p < 0.05$ was considered significant.

Results

Subject demographics

The baseline clinical characteristics and exacerbation status at the time of enrollment for all patients are shown in our previous report.^{13,14} Briefly, the mean age was 58.5 ± 12.1 (range: 29–83) years. Men comprised 42.9% ($n = 45$) and atopic patients constituted 61.9% ($n = 65$) of the study population. Eleven (10.5%) patients were

current smokers, and fifty-six (53.3%) were former smokers. Thirty-nine (37.1%) patients used oral corticosteroids (OCS) daily. Only five patients were treated with omalizumab, and none received anti-IL-5, anti-IL-5R, and anti-IL-4/13R antibodies during the follow-up period. Normality assumptions were met for the logarithm-transformed Eo and FeNO values. The geometric mean of Eo was 197.0 cells/ μ L and that of FeNO was 30.2 ppb (Table 1).

When the patients were classified according to their exacerbation status, the Eo and FeNO concentrations were significantly higher in the CFE group than in the other two groups (P for trend = 0.032 and 0.013, respectively; Table 2). However, the total serum IgE and serum periostin levels did not differ among the three groups.

Determination of the cutoff value of Eo and FeNO for the prediction of asthma exacerbation

ROC curves showed that the Eo cutoff value with the highest combination of sensitivity (73.3%) and specificity (56.7%) for all study patients was 250 cells/ μ L (Fig. 1A). Similarly, the cutoff value of FeNO with the highest combination of sensitivity (66.7%) and specificity (58.9%) was 31 ppb (Fig. 1B). The area under the curve was 0.627 for Eo and 0.612 for FeNO.

Baseline clinical characteristics according to the number of positive Th2 features

Based on the ROC curve analysis, we defined three parameters of "positive" Th2 features, as follows: 1) Eo $\geq$ 250 cells/ μ L; 2) FeNO $\geq$ 31 ppb; and 3) atopy, defined as the presence of a specific IgE for at least one common inhaled allergen. A Venn diagram revealed that there was a certain degree of overlap among the three positive Th2 features (Fig. 2). Among the 105 patients, 40 (38.1%) had one, 25 (23.8%) had two, and 24 (22.9%) had all three positive Th2 features (Fig. 3). Tables 3 and 4 show the clinical characteristics and biomarkers at baseline (VE), stratified according to the number of positive Th2 features. Patients with all three positive Th2 features displayed significantly higher Eo, FeNO and serum IgE levels than did the other patients (Table 4). In contrast, the number of positive Th2 features was not associated with pulmonary function, asthma control test (ACT), or asthma quality of life questionnaire (AQLQ) (Table 3).

Association of the number of positive Th2 markers with exacerbation frequency

Table 5 shows the frequency of frequent exacerbators and the OR in the four groups, in comparison with the patients with no

Table 2

Biomarkers according to the exacerbation status in three years.

	CNE (N = 39)	IE (N = 51)	CFE (N = 15)	P for trend
Asthma duration, years	16.8 $\pm$ 11.1	22.0 $\pm$ 16.5	19.3 $\pm$ 15.4	n/a
Maintenance OCS use, N (%)	13 (33.3%)	17 (33.3%)	9 (60.0%)	n/a
FEV1, % predicted	93.7 $\pm$ 18.0	89.9 $\pm$ 19.7	90.5 $\pm$ 19.5	0.529
Blood eosinophil, cells/ μ L	149.7 (0.52)	211.0 (0.52)	318.1 (0.45)	0.032
FeNO, ppb	23.1 (0.35)	34.3 (0.34)	39.0 (0.39)	0.013
Total serum IgE, IU/mL	163.9 (0.74)	127.4 (0.70)	118.8 (0.60)	0.301
Serum periostin, ng/mL	77.4 (0.22)	82.8 (0.20)	79.3 (0.21)	0.462

Data are shown as the geometric mean (log10 SD).

P-values were obtained using Jonckheere-Terpstra trend test.

CFE, consistent frequent exacerbators; CNE, consistent non-exacerbators; IE, intermittent exacerbators; n/a, not applicable.

positive Th2 features. The OR of the frequent exacerbators was 16.8 (95% CI: 1.13–247.8) in patients with all three positive Th2 markers compared with the patients who had no Th2 features ($p = 0.040$). The HR for the incidence of exacerbation in patients with two or three Th2 features were 1.37 (95% CI: 0.84–2.21; $p = 0.207$).

Discussion

Following our recent reports that showed the associations of Eo and FeNO levels with exacerbation status after a three-year follow-up in severe asthma patients, we aimed to determine the cutoff Eo and FeNO levels using ROC curve analyses, in order to make our previous results¹³ more clinically useful. Further, we proposed an estimated model using three Th2 biomarkers for better prediction of future asthma exacerbation.

In the current study, we attempted to determine the exact cutoff values using ROC curve analyses. However, we also agree that some may have skeptical opinions on the determination of the exact cutoff levels and their use in clinical settings. The non-essentiality would be due to the reliability of each biomarker and values that are too close to the cutoff levels. Thus, the follow-up strategy should be based on not only one value, but a comprehensive evaluation of several biomarkers, together with the results of a pulmonary function test and an understanding of the patient's symptoms. Our proposed model using three Th2 markers would thus be useful in providing better guidance for the management of severe asthma in the real world.

In this analysis, we intentionally selected three Th2 markers based on the following reasons. First, in our recent reports, Eo and FeNO levels appeared to be significantly different between frequent exacerbators and non-exacerbators.¹³ In addition, these parameters can be readily investigated in a less invasive manner. In contrast, we did not include serum IgE and periostin levels, which have been reported to have distinct potential, compared with other Th2 biomarkers, because the levels of these two markers were shown to be similar between frequent exacerbators and non-exacerbators.¹³ Further, periostin is not clinically available in our country. Instead, we included atopic status for analysis because its cutoff level has been established, and the presence of atopy is one of the criteria for the use of anti-IgE antibody.

Several cutoff levels of FeNO and Eo have been recommended in several guidelines and studies of asthma. These were determined on the basis of their intended purposes, including diagnosis, prognostication, and therapy.^{10,17,19,20} Moreover, it should be noted that the results of these analyses might have been largely

Table 1

Characteristics of the subjects at entry (N = 105).

Male sex, N (%)	45 (42.9%)
Age at enrollment, years	58.5 $\pm$ 12.1
Smoking status (Current/Ex/Never)	11/56/38
Pack years	5.5 (0–23.4)
Body mass index, kg/m ²	25.5 $\pm$ 5.0
Daily ICS dose, μ g [†]	1638 $\pm$ 518.8
Maintenance OCS use, N (%)	39 (37.1%)
Atopy, N (%)	65 (61.9%)
Omalizumab use, N (%)	5 (4.8%)
Blood eosinophil, cells/ μ L	197.0 (0.52)
Serum IgE, IU/mL	138.5 (0.70)
FeNO, ppb	30.2 (0.36)
FEV1, %predicted	91.4 $\pm$ 18.9
FEV1/FVC, %	66.3 $\pm$ 12.7

Data are shown as the mean $\pm$ standard deviation, median (interquartile range), geometric mean (log10 SD), or number (%).

[†] Equivalent to budesonide dose.

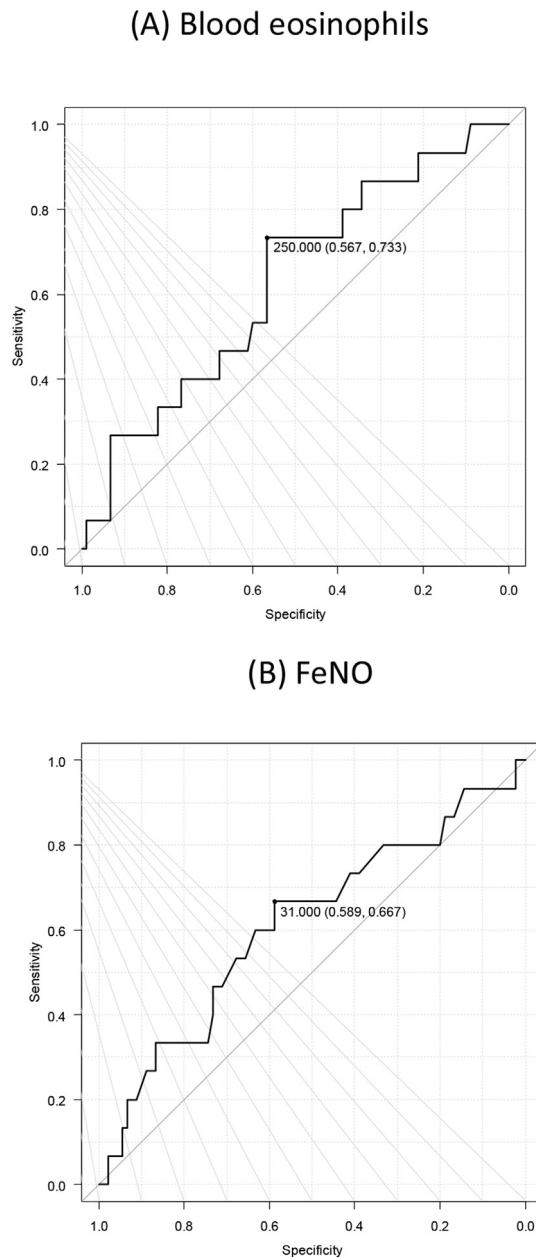


Fig. 1. Receiver operating characteristic (ROC) curves predicting frequent exacerbators (A) Blood eosinophil, (B) FeNO.

influenced by the characteristics of the population and the inclusion criteria of the study. In many studies on asthma, smokers, particularly heavy smokers, were excluded on the basis of the hypothesis that smoking may affect the characteristics of asthma.^{5,11,21–23} In the current study, we intentionally included smokers with severe asthma. Considering the high smoking rate in Japan and worldwide,²⁴ the inclusion of smokers in this study on severe asthma would more precisely reflect the actual clinical picture of severe asthma. Several reports have shown that smoking influences FeNO and Eo levels, suggesting that our strategy of including smokers provides data that reflects the actual clinical scenario. Further, our study was focused on only severe asthma, the pathogenesis of which remains unclear. Severe asthma also poses a significant economic and health burden. Recent significant advances in research have developed several biologics against high-Th2-related molecules, such as IgE, IL-5, or IL-4/13, which have

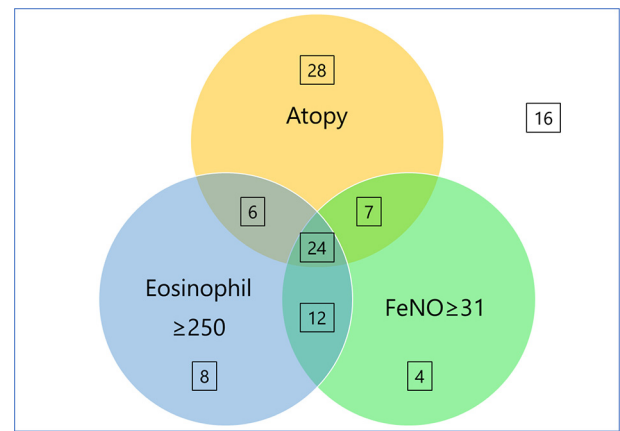


Fig. 2. Venn diagram showing the relationship among positive atopic status, high blood eosinophil counts (≥ 250 cells/ μ L), and high FeNO level (≥ 31 ppb).

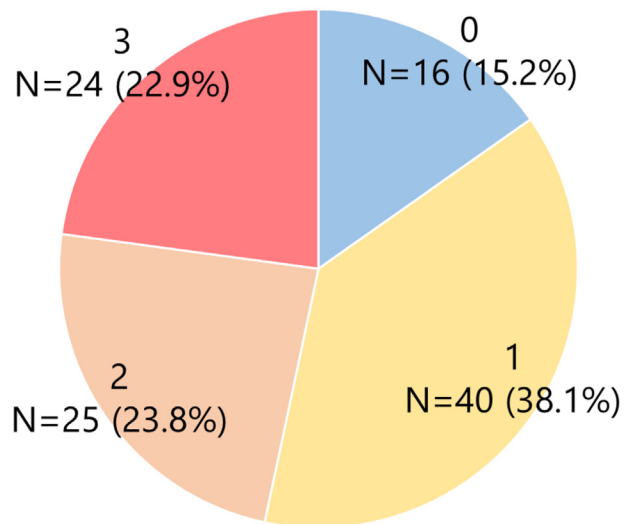


Fig. 3. Proportions of patients according to the number of positive Th2 features.

been demonstrated as possible treatment options for severe asthma.^{20,25–28} Accordingly, our results may provide better guidance on the initiation of these biologics.

Several biologics that target Th2-related molecules are well known to significantly affect the levels of Th2 markers.^{25,27,29} The anti-IL-5 antibody has been shown to significantly reduce Eo, independent of its efficacy. Based on ELISA, the anti-IgE antibody was shown to influence serum IgE levels. Furthermore, the anti-IL-4R antibody was shown to significantly affect FeNO levels. During the study period, anti-IL-5 or anti-IL-4R antibody was not administered to any subject because of its clinical unavailability in Japan. Therefore, we concluded that addressing these issues was not necessary. However, since these biologics are currently widely available in our country, the results of this study cannot be extrapolated to real-world clinical settings that include patients who have received some biologics. Meanwhile, we would like to emphasize that the results of this study would help in making the decision to initiate treatment with biologics, predict response, and subsequently reduce exacerbation frequency.

This study has some limitations. First, the sample size may have been too small, despite the recruitment of patients from 29 affiliated hospitals/pulmonary clinics. Nevertheless, all patients were carefully followed-up, and the final follow-up rate at the conclusion

Table 3

Characteristics according to the number of positive Th2 features.

Numbers of positive Th2 features	0 (N = 16)	1 (N = 40)	2 (N = 25)	3 (N = 24)	P-value
Male sex, N (%)	6 (37.5%)	19 (47.5%)	8 (32.0%)	12 (50.0%)	0.524
Age at enrollment, years	64.0 ± 11.1	58.0 ± 13.2	59.5 ± 11.4	54.5 ± 10.4	0.104
Smoking status (Current/Ex/ Never)	3/9/4	2/25/13	2/8/15	4/14/6	0.072
Pack years	16.3 (2.0–23.6)	5.8 (0–30.4)	0 (0–11.9)	10.8 (1.13–26.8)	0.101
Body mass index, kg/m ²	25.3 ± 4.0	25.6 ± 4.8	26.1 ± 6.5	24.7 ± 4.2	0.794
Daily ICS dose, µg [†]	1843.8 ± 547.7	1505.6 ± 387.7	1662.0 ± 533.3	1700 ± 636.6	0.137
Maintenance OCS use, N (%)	8 (50.0%)	12 (30.0%)	9 (36.0%)	10 (41.7%)	0.526
Atopy, N (%)	0 (0%)	28 (70.0%)	13 (52.0%)	24 (100%)	<0.001
Omalizumab use, N (%)	3 (18.8%)	1 (2.5%)	0 (0%)	1 (4.17%)	0.035
FEV1, %predicted	92.0 ± 21.1	95.1 ± 17.0	90.0 ± 18.6	86.2 ± 20.6	0.320
FEV1/FVC, %	68.1 ± 13.2	67.6 ± 13.5	64.9 ± 11.1	64.4 ± 12.9	0.683
ACT	22.0 (17.5–23.0)	20.0 (17.0–23.0)	20.0 (15.8–23.0)	22.0 (16.5–23.5)	0.884
AQLQ	5.1 (4.7–5.9)	5.7 (4.9–6.4)	5.5 (4.9–6.2)	5.7 (4.6–6.3)	0.541

Data are shown as the mean ± standard deviation, median (interquartile range), or number (%).

P-values were obtained using a chi-square test, one-way analysis of variance (ANOVA), or the Mann–Whitney U-test, as appropriate.

ACT, asthma control test; AQLQ, asthma quality of life questionnaire.

[†] Equivalent to budesonide dose.**Table 4**

Biomarkers according to the number of positive Th2 features.

Numbers of positive Th2 features	0 (N = 16)	1 (N = 40)	2 (N = 25)	3 (N = 24)	P-value
Blood eosinophil, cells/µL	48.8 (0.44)	125.6 (0.37)	382.3 (0.44)	530.2 (0.21)	<0.0001
FeNO, ppb	12.6 (0.22)	19.7 (0.22)	46.3 (0.28)	70.2 (0.21)	<0.0001
Total serum IgE, IU/mL	30.1 (0.71)	132.3 (0.65)	151.2 (0.64)	377.8 (0.49)	<0.0001

Data are shown as the geometric mean (log₁₀ SD).

P-values were obtained using one-way analysis of variance (ANOVA).

Table 5

Association between the numbers of positive Th2 features and frequent exacerbation.

Numbers of positive Th2 features	Proportion of frequent exacerbators [†]	OR	95% CI	P-value
0	6.3%	Ref.		
1	10%	1.93	0.16–23.1	0.604
2	16%	12.8	0.83–196.8	0.068
3	25%	16.8	1.13–247.8	0.040

Logistic regression analysis.

Adjusted for age, sex, asthma duration, BMI, smoking status, Pack-year, sinus CT score, oral corticoid use and AERD.

[†] Two or more exacerbations per one year, over three years.

of the third year was high. Second, we did not include the frequency of emergency department (ED) visits as a criterion for asthma exacerbation. In Japan, pulmonary physicians often prescribe standby oral steroids for exacerbations. Therefore, the frequency of ED visits may not necessarily indicate the actual frequency of exacerbations. Lastly, we used the older definition of severe asthma that was developed during the ATS workshop in 2000,¹⁵ because the new definition reported in the ERS/ATS guidelines¹⁶ had not been officially announced when this study was designed. Nevertheless, as mentioned in the methods section, we were certain that all patients were under the appropriate treatment of high-dose inhaled corticosteroids or OCS for asthma control.

In conclusion, based on three follow-up timepoints, we determined the Eo and FeNO cutoff levels that may be used for predicting asthma exacerbation. The combined use of three Th2 features

would also be useful in guiding the decision on treatment strategy for severe asthma.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.alit.2020.09.001>.

Conflict of interest

MN received research funding from AstraZeneca, Kyorin Pharmaceutical and MSD. The rest of the authors have no conflict of interest.

Authors' contributions

HirokaK, statistical analysis, acquisition of data, interpretation of data, and drafting the manuscript; HM, study concept and design, interpretation of data, and critical revision of the manuscript; NaT, study concept and design, acquisition of data, interpretation of data, and critical revision of the manuscript; KS, HirokaK, acquisition of data and interpretation of data, and critical revision of the manuscript; NoT, MM, HG, MS, interpretation of data, and critical revision of the manuscript; MN, study concept and design, interpretation of data, and finalizing of the manuscript. SK, study concept and design, acquisition of data, interpretation of data, and finalizing of the manuscript.

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Determination of the cutoff values of Th2 markers for the prediction of future exacerbation in severe asthma: an analysis from the Hokkaido Severe Asthma Cohort Study

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Supplementary Methods

This study was approved by the ethics committees of Hokkaido University Hospital and its 29 affiliated hospitals and clinics, and all subjects provided their written informed consent. This study was registered in the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) system (https://upload.umin.ac.jp/cgi-open-bin/ctr/ctr_view.cgi?recptno=R000003917; ID no. 000003254). The details of the Materials and Methods employed in this study are described in our previous reports ¹.

1. Participants

We enrolled patients who were diagnosed with severe asthma by respiratory physicians between February 2010 and September 2012 at the Hokkaido University Hospital and its 29 affiliated hospitals and clinics. A total of 141 subjects with severe asthma underwent procedural evaluations during a two-day stay at the Hokkaido University Hospital. After confirmation of the inclusion criteria, adherence to medications, and inhalation technique, a total of 127 patients with severe asthma were selected for baseline analyses ¹. The study inclusion and exclusion criteria were described in our previous report ¹. Several clinical parameters were evaluated in all subjects during their two-day stay at Hokkaido University Hospital ¹.

2. Definition of asthma and severe asthma

The diagnosis of asthma was made by respiratory physicians according to the Global Initiative for Asthma (GINA) criteria ² on the basis of recurrent respiratory symptoms with demonstrable reversible airflow limitation. At some time during the course, variable airflow limitation was confirmed based on excessive bronchodilator reversibility or/and

significant increase in forced expiratory volume in one second (FEV₁) after treatment with controller medications (i.e., an increase in FEV₁ > 12% and > 200 mL). Severe asthma was diagnosed based on the American Thoracic Society (ATS) criteria of refractory asthma published in 2000 ³, with slight modifications, as follows:

Major Characteristics

In order to achieve asthma control:

1. Treatment with continuous or near continuous (>50% of year) oral corticosteroids
2. Requirement for treatment with high-dose inhaled corticosteroids: the inhaled corticosteroid doses were modified due to differences in their availability across Japan, as follows: Flutide® ≥800 µg, Pulmicort® ≥1200 µg, QVAR® ≥600 µg, Alvesco® ≥600 µg, Asmanex® ≥600 µg, Adoair® ≥1000 µg, and Symbicort® ≥960 µg.

Minor Characteristics

1. Requirement for daily treatment with a controller medication in addition to inhaled corticosteroids, e.g., long-acting β-agonist, theophylline, or leukotriene antagonist
2. Asthma symptoms requiring short-acting β-agonist use on a daily or near daily basis
3. Persistent airway obstruction (FEV₁<80% predicted; diurnal PEF variability>20%)
4. One or more urgent care visits for asthma per year
5. Three or more oral steroid “bursts” per year
6. Prompt deterioration with<25% reduction in oral or inhaled corticosteroid dose
7. Near fatal asthma event in the past

The diagnosis of severe asthma required one or both major criteria and two minor criteria. Patients who were well-controlled under the current medications (absence of any

of the minor characteristics 2, 4, or 5 upon study entry) were asked if within one year they experienced episodic deterioration of symptoms, urgent care visits, and rescue use of short-acting bronchodilators when the dose of the current medication was reduced.

3. Clinical evaluations

Upon entry to the study and admission to Hokkaido University Hospital, all subjects underwent evaluation for several clinical parameters.

3-1. Pulmonary function test

Upon study entry and during each hospital visit, spirometry was performed before and after inhalation of 400 µg oxitropium and 400 µg salbutamol. The maximum forced expiratory volume in one second (FEV₁) obtained from the two procedures and the FEV₁/FVC (forced vital capacity) value that corresponded to the maximum FEV₁ value were used for the analyses. Owing to the severity of asthma in all subjects, respiratory medicines were not prohibited prior to the procedure, except for the use of short-acting bronchodilators for at least 12 hours prior to all measurements. Pulmonary function tests were performed, as described in our previous report ¹.

3-2. Questionnaires

Clinical research coordinators (CRCs) directly administered comprehensive questionnaires to all subjects on day 1 of admission. The questionnaires were administered to obtain the following information:

- i. Age at the onset of asthma

The age of the patient at the onset of asthma was self-reported. To ensure accuracy, patients were asked to describe their experiences during episodes of dyspnea, wheezing, or coughing during childhood and puberty. In cases of uncertainty, the time of the earliest respiratory symptoms was designated as the age of onset of asthma symptoms and was used to calculate the duration of asthma.

ii. Smoking status

The smoking status of all patients was assessed by the CRCs. We classified subjects into two groups: smokers [≥ 10 pack-years (PY)] and nonsmokers (< 10 PY), according to the number of cigarette packs based on our previously reports ¹.

iii. Asthma quality of life questionnaire

In all participants, the asthma-related quality of life (QOL) was assessed using the Japanese version of the Asthma Quality of Life Questionnaire (AQLQ) developed by Juniper ^{4, 5}. The AQLQ consisted of 32 items separated into four sections: symptoms, activity limitations, emotional function, and exposure to environmental stimuli. The overall score of the AQLQ was calculated as the mean of all items. Permission to use the Japanese version of the AQLQ was obtained from Dr. Juniper.

3-3. Measurement of biomarkers

The peripheral blood eosinophil count was obtained from the standard complete blood count, which was performed by the Laboratory Division of Hokkaido University Hospital. Specific IgE responses to common inhaled allergens, including *Dermatophagoides farinae*, grass pollens, animal dander, and molds, were determined. Atopy was defined

based on positive multiple-antigen simultaneous test scores (lumicount > 1.01) to at least one common inhaled allergen^{1, 6}. Blood and sputum supernatant samples were immediately frozen after collection and stored at -80°C until assay.

4. Protocol of the three-year follow-up

4-1. Assessment of exacerbation

We gave all participants an “asthma diary” to collect exacerbation information. When systemic corticosteroids for an asthma attack were administered in the hospitals/clinics, we asked the doctors to record in the patient’s asthma diary the dose and the total number of days in which systemic steroids were used. When oral corticosteroids (OCS) for an asthma attack were used by the patients themselves at home, the patients were instructed to record this information their diaries. In this case, the chief doctor carefully checked the diary during each patient’s visit to the hospital/clinic to determine if their asthma was worsening. During their yearly visits to the Hokkaido University Hospital, the patients were directly questioned by the clinical research coordinator (CRC) and a doctor regarding the number of exacerbation episodes within one year, and the CRC/doctor confirmed the patients’ responses in the diary records.

4-2. Assessment of adherence to medications

During the follow-up period, we asked all subjects to record their medication use in their asthma diary for four weeks every season (a total of 16 weeks per year). We also asked the patients to send their diaries to the Hokkaido Respiratory Cohort Office for verification by the CRCs. The patients visited each hospital/clinic every 1-2 months and the chief doctor confirmed adherence to their medications at every visit. For the patients

who visited Hokkaido University once a year, the CRCs and doctors (Dr. HK, SK, KS, YM, HK) directly inquired regarding the patients' adherence.

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