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Original Article

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Mepolizumab decreased the levels of serum galectin-10 and eosinophil cationic protein in asthma

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ABSTRACT

Background: Mepolizumab, a humanized antibody targeting interleukin-5, decreases the number of blood eosinophils and the frequency of exacerbation of severe asthma. Galectin-10 is a protein within the cytoplasm of eosinophils and constitutes Charcot-Leyden crystals, which promotes key features of asthma. However, the relationship between time kinetics and clinical response of eosinophil-derived molecules such as galectin-10 or eosinophil cationic protein (ECP) has not been precisely investigated.
Objective: This study aimed to clarify the precise time course of the levels of serum galectin-10 and ECP after mepolizumab treatment and to analyze the relationship between the levels of eosinophil-derived molecules and the clinical background or response to mepolizumab treatment.
Methods: This multicenter, prospective open-label study recruited 20 patients with severe eosinophilic asthma. Mepolizumab was administered every 4 weeks for 32 weeks and the levels of various biomarkers were serially analyzed.
Results: The serum galectin-10 and ECP significantly and rapidly decreased 4 weeks after initial administration of mepolizumab. In contrast, basophil count, fractional exhaled nitric oxide, and the serum total IgE level were unchanged during treatment. Asthma Control Questionnaire-5, Asthma Health Questionnaire-33, and Lund-Mackay scores significantly improved after mepolizumab treatment. Both high ECP and eosinophil count related to better response in forced expiratory volume in 1 second (FEV_1) and measurable ECP level at 4 weeks after administration of mepolizumab related to the further improvement in FEV_1 toward week 32. No significant difference in improvement in FEV_1 was observed in galectin-10 high group. The level of ECP at baseline was significantly related to the higher prevalence of nasal polyp and Lund-Mackay score.

Conclusion: This study was the first to show that the levels of serum galectin-10 decreases after initial administration of mepolizumab. The significant relationship between serum ECP and better response in FEV_1 suggested the potential role of serum ECP as a predictive biomarker for the efficacy of mepolizumab (UMIN000030466).

Keywords: Asthma; Eosinophil cationic protein; Galectin-10; Interleukin-5; Mepolizumab

Conflict of Interest

Konomi Kobayashi: None. Hiroyuki Nagase: Lecture fees: GlaxoSmithKline, AstraZeneca, Novartis Pharma, Sanofi. Naoya Sugimoto: None. Shiho Yamamoto: None. Akihiko Tanaka: Lecture fees: GlaxoSmithKline, AstraZeneca, Novartis Pharma, Sanofi. Koichi Fukunaga: Lecture fees: GlaxoSmithKline, AstraZeneca, Novartis Pharma, Sanofi. Ryo Atsuta: Lecture fees: GlaxoSmithKline, AstraZeneca, Novartis Pharma, Sanofi. Etsuko Tagaya: Lecture fees: GlaxoSmithKline, AstraZeneca, Novartis Pharma, Sanofi. Masayuki Hojo: Lecture fees: GlaxoSmithKline, AstraZeneca, Novartis Pharma, Sanofi. Yasuhiro Gon: Lecture fees: GlaxoSmithKline, AstraZeneca, Novartis Pharma, Sanofi.

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Conceptualization: Hiroyuki Nagase, Akihiko Tanaka, Koichi Fukunaga, Ryo Atsuta, Etsuko Tagaya, Masayuki Hojo, Yasuhiro Gon. Formal analysis: Konomi Kobayashi, Hiroyuki Nagase. Investigation: Konomi Kobayashi, Hiroyuki Nagase, Naoya Sugimoto, Shiho Yamamoto, Yasuhiro Gon. Methodology: Hiroyuki Nagase, Akihiko Tanaka, Koichi Fukunaga, Ryo Atsuta, Etsuko Tagaya, Masayuki Hojo, Yasuhiro Gon. Project administration: Hiroyuki Nagase. Writing - original draft: Konomi Kobayashi, Hiroyuki Nagase. Writing - review & editing: Konomi Kobayashi, Hiroyuki Nagase.

INTRODUCTION

Interleukin-5 (IL-5) is among the most eosinophil-specific and potent activators of various cellular functions such as production, activation, and maturation of eosinophils [1]. Mepolizumab is a humanized mAb targeting IL-5 that decreases the number of blood eosinophils and the frequency of exacerbation of severe eosinophilic asthma [2].

Galectin-10 is a protein within the cytoplasm of eosinophils. Activated eosinophils induce the loss of cytoplasmic localization of galectin-10, leading to the formation of Charcot-Leyden crystals (CLC) during extracellular trap cell death (ETosis) [3]. Furthermore, recent evidence using mouse models suggested that CLCs promote the key features of asthma pathology [4]. Despite the increase of galectin-10 in sputum of patients with asthma [5], the usefulness of serum galectin-10 as a biomarker of asthma has not been established and its time course after mepolizumab treatment remains unknown.

Eosinophil cationic protein (ECP) is also an eosinophil-derived molecule stored in granules, which are released during inflammation and induce tissue damage in asthmatic airways. Several studies have shown that the expression of ECP is higher in symptomatic than asymptomatic patients with asthma and this is related to the severity of asthma [6]. However, the precise time course of serum ECP after mepolizumab treatment at clinically approved dose has not been analyzed in real-world settings.

Although the time course of eosinophil count after mepolizumab treatment has been precisely investigated, eosinophil-derived molecules such as galectin-10 and ECP may reflect the activity of eosinophils, and information on their time course could help in understanding the mechanism of mepolizumab.

Thus, this study aimed to clarify the precise time course of galectin-10 and ECP levels after initiating mepolizumab treatment. We serially analyzed the levels of those molecules in serum obtained from patients with severe asthma treated with mepolizumab for 32 weeks. As the number of blood eosinophils has been established as a predictive marker for treatment response to mepolizumab [7], we also attempted to determine the relationship between the levels of galectin-10 and ECP and their impact to pulmonary function and symptoms during mepolizumab treatment.

MATERIALS AND METHODS

Study design and patient population

This multicenter, prospective open study investigated patients with asthma aged at least 18 years who had never treated with mepolizumab previously. We included patients with a

score of at least 0.75 in the 5-item Asthma Control Questionnaire (ACQ-5) during treatment comprising at least 400 μg of fluticasone propionate or the equivalent by inhalation per day and at least 3 months of treatment with an additional controller; those who experienced at least one occurrence of asthma exacerbation that required more than 3 days of systemic corticosteroids, Emergency Department visit, or an admission in the previous year; and blood eosinophil count of at least 150 cells/μL at screening or at least 300 cells/μL at some time during the previous year. Patients with past smoking history were included, and presence of forced expiratory volume in 1 second (FEV_1) reversibility was not necessary. This study was approved by Teikyo University Ethical Review Board for Medical and Health Research Involving Human Subjects (16–159) and also by ethics committees at each institution, and all patients provided written informed consent.

A total of 20 patients started treatment with mepolizumab from January 2017 until December 2017 at Teikyo University Hospital (n = 15) and Nihon University Hospital (n = 5). Although one patient stopped the administration at week 24, all other patients completed the study. The baseline characteristics of the patients are shown in **Table 1**. Smoking index is the product of the number of cigarettes smoked per day and years of smoking.

Study intervention and measurements

The patients were administered 100 mg of mepolizumab subcutaneously every 4 weeks for 32 weeks. During the study period, the doses of inhaled or oral corticosteroids were fixed at the initial dose. At each visit, patients recorded scores of ACQ-5, Asthma Control Test (ACT), and Asthma Health Questionnaire (AHQ)-33 [8], to estimate their quality of life (QoL) and underwent hematologic tests. Pulmonary function testing and measurement of fractional exhaled nitric oxide (FeNO) by NIOX VERO (Circassia AB, Uppsala, Sweden) were performed before starting mepolizumab (week 0) and after 4, 12, and 32 weeks. Airway reversibility was defined as positive when FEV_1 increase 200 mL and 12% after inhalation of bronchodilator. Computed tomography (CT) scans of paranasal sinuses were evaluated prior to the first injection of mepolizumab and at 12 and 32 weeks.

Total white blood cell count and percentage of eosinophils and basophils were measured by an automated hematology analyzer. The levels of serum galectin-10 were measured by enzyme-linked immunosorbent assay kit (Thermofisher, EH204RB, Carlsbad, CA, USA). ECP was measured by fluorescence enzyme immunoassay using serum collected from blood samples centrifuged after leaving for 60 minutes at 25°C. The measurable range of ECP was 2–200 μg/L and the normal limit of ECP in healthy adults was below 14.9 μg/L [9]. Regarding the value at baseline, patients were stratified into high or low groups by median values of ECP (12.3 μg/L), eosinophil counts (403/μL), and galectin-10 (23.1 ng/mL).

The diagnosis of eosinophilic chronic rhinosinusitis (ECRS) was based on the Japanese Epidemiological Survey of Refractory Eosinophilic Chronic Rhinosinusitis (JESREC) scoring system [10], which includes the presence of nasal polyps (either unilateral or bilateral), peripheral blood eosinophil count, and dominant shadow of ethmoid sinuses shown in the CT scan of paranasal sinuses. A JESREC score higher than or equal to 11 indicated ECRS and did not require biopsy. CT findings of paranasal sinuses were staged by Lund-Mackay (LM) scores [11], ranging from 0 to 24, based on the findings of each paranasal sinus and obstruction of ostiomeatal complex on each side.

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Table 1. Baseline characteristics of patients

Characteristic	ECP		Eosinophil count		Galectin-10		Total (n = 20)
	High (n = 9)	Low (n = 10)	High (n = 10)	Low (n = 10)	High (n = 8)	Low (n = 8)	
Female sex (%)	77.8	60.0	90.0	50.0	75.0	62.5	70.0
Age (yr)	53.6±5.1	61.2±4.9	55.6±4.9	59.5±4.9	57.6±5.2	51.3±5.2	57.6±3.4
Onset age (yr)	32.2±7.3	40.4±7.0	31.3±6.7	41.9±6.7	39.0±7.7	32.1±7.7	36.6±4.7
BMI (kg/m^2)	25.9±1.4	25.1±1.3	24.9±1.3	25.5±1.3	23.7±1.5	26.7±1.5	25.2±0.9
Ex/never smoker (%)	44.4/55.6	40.0/50.0	30.0/70.0	50.0/40.0	12.5/75.0	50.0/37.5	90.0/100.0
Smoking Index†	123±128	301±128	71±113	346±120	20±136	381±145	201±80
Atopic (%)	55.6	50.0	50.0	50.0	37.5	50.0	50.0
ACT	14.7±1.8	13.8±1.8	15.3±1.6	12.7±1.7	15.1±2.0	14.0±2.0	14.4±1.2
ACQ-5	2.27±0.41	2.56±0.39	2.44±0.39	2.48±0.39	2.53±0.46	2.38±0.46	2.46±1.20
Previous exac./yr	3.1±1.8	4.3±1.6	3.3±1.6	4.1±1.6	5.8±1.9	2.0±2.0	3.8±1.2
Eosinophil count (/μL)	802±86**	230±82	933±105**	232±105	768±175	391±175	572±110
ECP (μg/L)	30.7±3.8**	6.4±3.6	29.5±4.2**	7.4±4.0	21.2±6.7	13.8±6.2	18.0±3.8
Galectin-10 (ng/mL)	38.4±9.1	15.6±8.5	49.8±11.5	15.6±11.5	53.5±10.6*	11.9±10.6	32.7±9.0
IgE (IU/mL)	339±137	308±130	304±128	374±128	214±144	470±144	339±89
FeNO (ppb)	86.4±21.3	27.6±19.1	84.9±20.0	30.0±19.0	49.4±28.0	71.5±26.1	55.9±14.9
Basophil count (/μL)	43.1±8.3	35.2±7.9	40.3±8.2	33.7±8.2	32.8±8.7	37.9±8.7	37.0±5.7
Nasal polyp (%)	77.8**	0.0	60.0	10.0	37.5	28.6	35.0
ECRS (%)	77.8	40.0	80.0	40.0	62.5	50.0	60.0
JESREC	13.6±1.7*	7.5±1.6	12.7±1.6	8.1±1.6	10.8±1.9	9.9±1.9	10.4±1.3
LM score	12.8±2.0**	4.3±1.9	11.2±2.0	5.3±2.0	10.5±2.5	6.4±2.5	8.3±1.6
AERD (%)	22.2	10	20	10	12.5	25	15
ICS (μg/day)	1,100±86	1,015±82	1,150±87	1,015±87	1,113±113	1,094±113	1,083±62
LABA (%)	100	100	100	100	100	100	100
LAMA (%)	44.4	70.0	40.0	80.0	50.0	62.5	60.0
OCS (%)	22.2	30.0	20.0	30.0	25.0	37.5	25.0
OCS dose (mg/day)	15.0±4.1	10.0±3.3	15.0±4.1	10.0±3.3	10.0±4.4	13.3±3.6	12.0±2.6
%FEV$_1$ (%)	84.1±10.0	94.0±9.5	87.6±9.4	89.5±9.4	93.7±10.4	85.7±10.4	88.6±6.5
FEV$_1$ (L)	2.01±0.29	2.07±0.28	1.94±0.27	2.09±0.27	2.04±0.29	2.26±0.29	2.02±0.19
Reversibility (mL)	237±52*	53±55	149±61	114±68	111±56	105±64	133±44
Reversibility positive (%)	33.3	0.0	20.0	12.5	12.5	16.7	16.7
%V25	26.0±10.4	37.8±9.9	28.3±10.5	35.7±10.0	39.7±13.1	32.1±12.3	32.2±7.1
R5-R20	0.47±0.23	1.05±0.23	0.58±0.23	0.99±0.26	0.38±0.24	0.80±0.21	0.76±0.17
ALX	9.4±4.3	8.5±4.3	11.2±4.0	6.1±4.5	5.4±2.3	5.3±2.0	9.0±2.9

Values are presented as the mean ± standard error of the mean unless otherwise indicated.
BMI, body mass index; ACT, Asthma Control Test; ACQ, Asthma Control Questionnaire; ECP, eosinophil cationic protein; IgE, immunoglobulin E; FeNO, fractional exhaled nitric oxide; ECRS, eosinophilic chronic rhinosinusitis; JESREC, Japanese Epidemiological Survey of Refractory Eosinophilic Chronic Rhinosinusitis; LM score, Lund-Mackay score; AERD, aspirin-exacerbated respiratory disease; ICS, inhaled corticosteroid; LABA, long-acting beta2 agonist; LAMA, long-acting muscarinic antagonist; OCS, oral corticosteroid; FEV$_1$, forced expiratory volume in 1 second.
*p < 0.05, **p < 0.01 vs. each low groups, †Number of cigarettes smoked per day × years of smoking.

Statistical analysis

Bartlett test was employed to check the variances across samples. Differences between the 2 groups were analyzed using Student *t* test or Mann-Whitney *U* test when the variances were equal or different, respectively. The relationship between biomarkers was analyzed by Spearman correlation coefficient. The clinical index and biomarkers at different time points were analyzed by 2-way analysis of variance followed by Dunnett multiple comparisons test. Data were presented as mean ± standard error of the mean. JMP 14 (SAS Institute Japan, Tokyo, Japan) or GraphPad Prism ver. 8 (GraphPad Software, San Diego, CA, USA) was used for statistical analyses. Statistical significance was defined as $p < 0.05$.

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RESULTS

A total of 20 patients initiated mepolizumab treatment. At baseline, the mean scores of ACT and ACQ-5 were 14.4 and 2.46, respectively, both representing uncontrolled asthma (**Table 1**). The frequency of previous exacerbation before mepolizumab treatment was 3.8/yr. We stratified patients into 2 groups by median values of ECP, blood eosinophil count, and galectin-10. There were no significant differences in ACT score, ACQ-5 score, or the frequency of previous exacerbation between high and low groups of each biomarker. As for comorbidities, all patients with nasal polyp belonged to high ECP ($p < 0.01$). Patients with high eosinophil count or ECP tended to have ECRS more frequently than those with low counts. In addition, JESREC score or LM score was significantly higher in the high ECP group; however, the difference did not reach statistical significance in high eosinophil or galectin-10 group, suggesting that the activities of nasal comorbidities are highly reflected to levels of serum ECP. Patient characteristics were not significantly different between patients with high and low galectin-10 groups.

Next, we analyzed the time course of clinical index during administration of mepolizumab for 32 weeks. ACT score and ACQ-5 significantly improved at 4 weeks (**Fig. 1A, B**), and the improvements were maintained until 32 weeks. The QoL shown by AHQ score also significantly

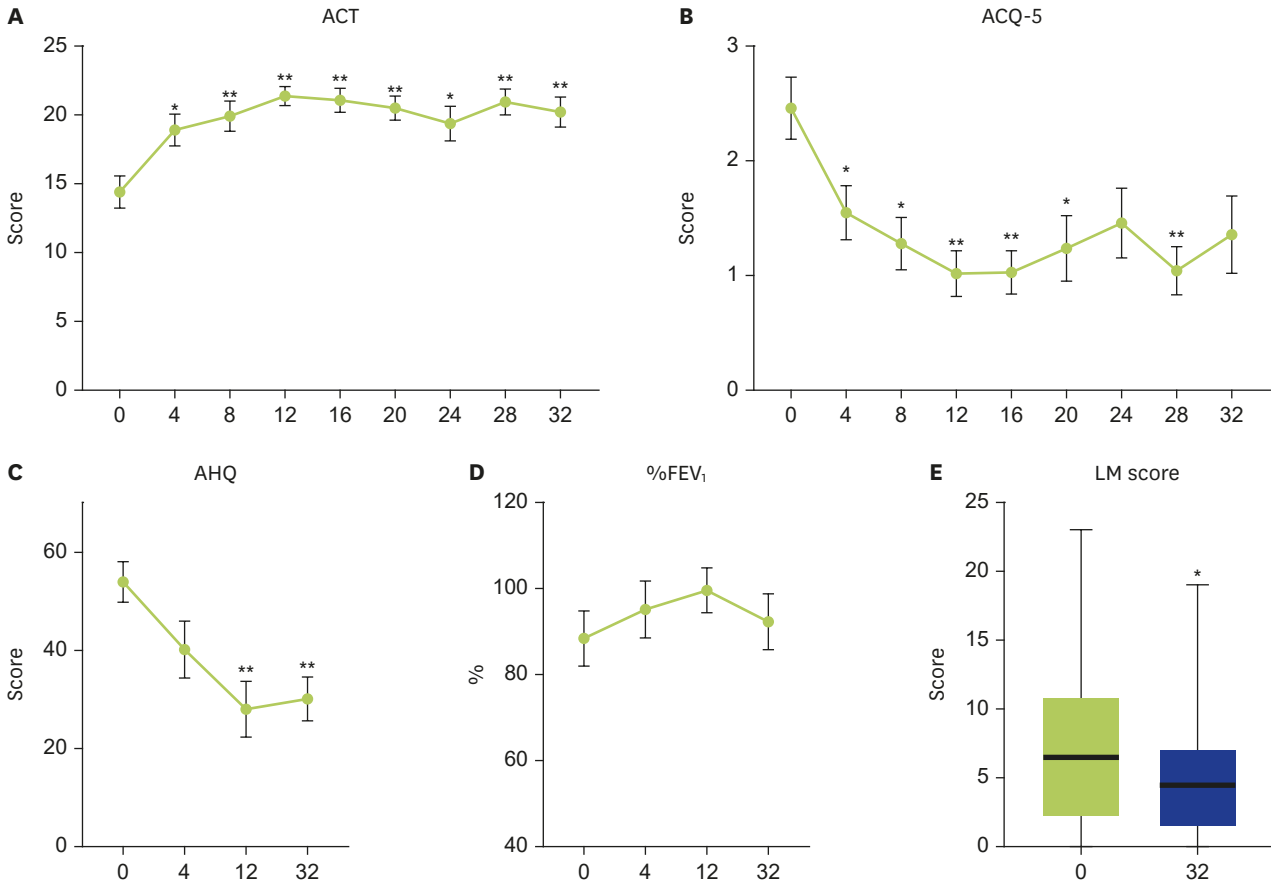

Fig. 1. Time course of clinical index during mepolizumab therapy. Values of Asthma Control Test (ACT; A), Asthma Control Questionnaire (ACQ)-5 (B), Asthma Health Questionnaire (AHQ)-33 (C), forced expiratory volume in 1 second, % predicted (%FEV_1; D) are presented as the mean ± standard error of the mean. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. data at baseline (week 0). (E) The boxes represent medians and bars represent minimum to maximum values of Lund-Mackay (LM) scores.

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improved after 12 weeks treatment (**Fig. 1C**). Although $FEV_{1\%}$predicted did not significantly improve in the overall analysis (**Fig. 1D**), LM score significantly improved after treatment for 32 weeks (**Fig. 1E**). The frequency of exacerbation numerically decreased to 3.7 ± 6.0/yr.

We further analyzed the time course of biomarkers during mepolizumab treatment (**Fig. 2**). Mepolizumab significantly reduced the levels of eosinophil count, ECP, and serum galectin-10 by week 4 and maintained at low level through week 32 (**Fig. 2A–C**). The mean blood eosinophil count at baseline was 571.7/μL and the count significantly correlated with ECP (R^2 = 0.753, p < 0.01) and galectin-10 (R^2 = 0.363, p < 0.05). However, the significant correlation was not observed in between ECP and galectin-10. In contrast, basophil count, FeNO, and total serum IgE did not show a significant change during mepolizumab treatment (**Fig. 2D–F**).

Subsequently, we analyzed the relationships between the baseline value of eosinophil count, ECP, and galectin-10 and the clinical response after 32 weeks of treatment. Although there were no significant differences in the improvement of ACT, ACQ-5, AHQ, and LM score based on the baseline values of those markers (**Table 2**), patients in all groups showed the improvement in ACQ-5 above 0.5 point of minimal clinically important difference. The improvement in LM score tended to be higher in the high ECP group (p = 0.063).

However, the improvement in FEV_1 (mL) was significantly higher in the group with high ECP (**Table 2**, **Fig. 3A**) or eosinophil counts (**Fig. 3B**) than those with low values. The FEV_1 was

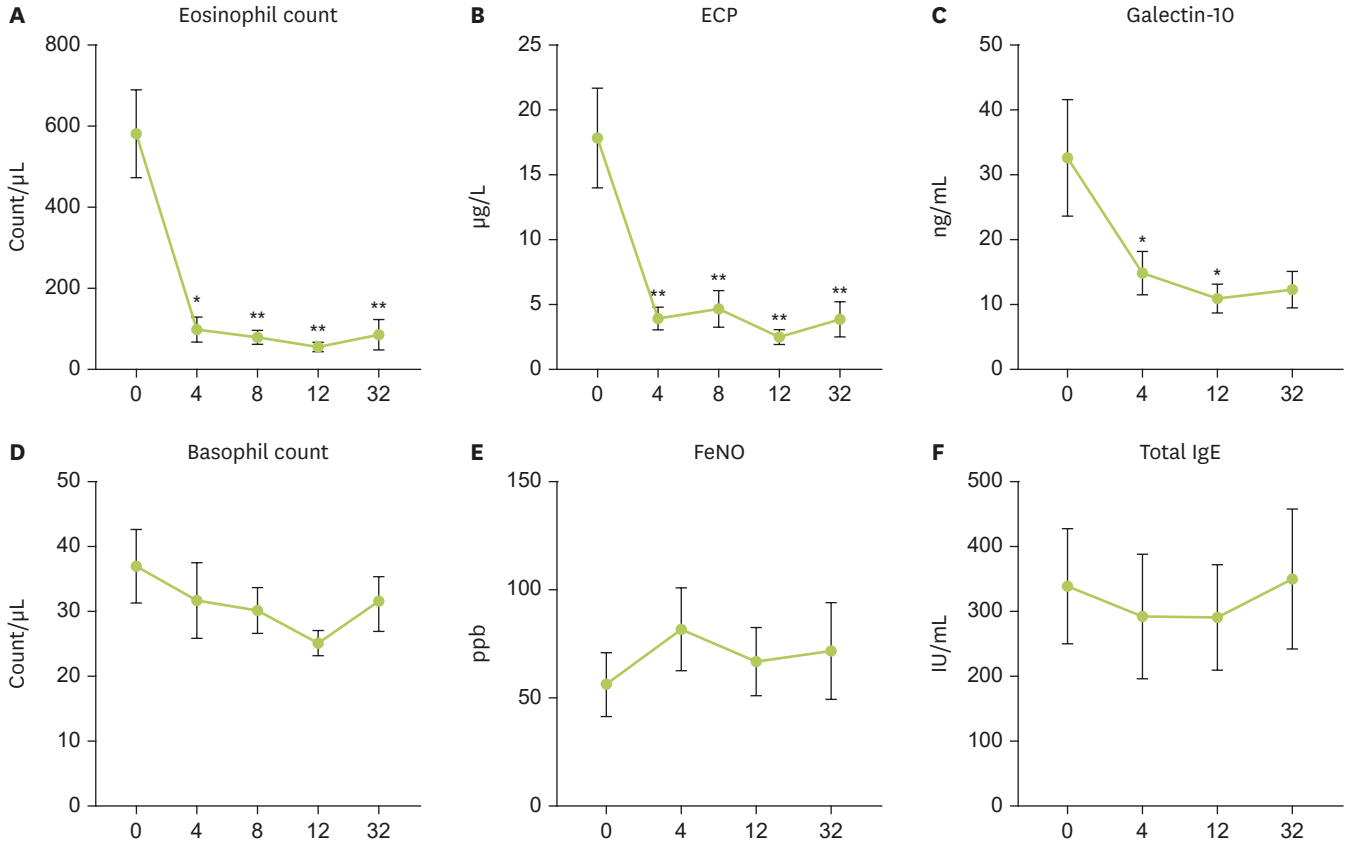

Fig. 2. Time course of biomarkers during mepolizumab therapy. Values of eosinophil count (A), serum eosinophil cationic protein (ECP; B), serum galectin-10 (C), blood basophil count (D), fractional exhaled nitric oxide (FeNO; E), and total serum immunoglobulin E (IgE) (F) are presented as the mean ± standard error of the mean. $^{*}p$ < 0.05, $^{**}p$ < 0.01 vs. data at baseline (week 0).

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numerically higher in high ECP or eosinophil count group throughout the treatment period (**Fig. 3C, D**). Regarding galectin-10, no significant difference in improvement in FEV_1 was observed between groups.

Table 2. Differences in clinical responses at 32 weeks stratified by baseline value of biomarkers

Variable	ECP		Eosinophil Count		Galectin-10		Total (n = 20)
	High (n = 9)	Low (n = 10)	High (n = 10)	Low (n = 10)	High (n = 8)	Low (n = 8)	
ΔACT	4.6±2.1	6.5±2.1	4.7±1.9	7.5±2.2	4.5±2.4	6.5±2.8	5.9±1.4
ΔACQ-5	−0.73±0.51	−1.73±0.54	−1.10±0.51	−1.58±0.57	−1.18±0.60	−1.4±0.69	−1.31±0.37
ΔAHQ	−23.2±8.4	−24.1±8.4	−24.0±8.1	−27.0±8.6	−25.9±7.7	−23.4±8.3	−25.4±5.8
%ΔFEV_1 (%)	14.4±6.4	−3.3±6.1	18.5±5.6**	−5.2±5.6	7.5±6.1	−4.5±6.1	6.7±4.8
ΔFEV_1 (mL)	260±103*	−98±98	317±99**	−106±99	189±119	−116±119	106±85
ΔLM score	−4.4±1.3	−0.9±1.2	−3.4±1.3	−1.8±1.3	−2.5±1.5	−1.6±1.4	−2.6±0.9

Values are presented as the mean ± standard error of the mean.
ACT, Asthma Control Test; ACQ, Asthma Control Questionnaire; AHQ, Asthma Health Questionnaire; FEV_1, forced expiratory volume in 1 second; LM score, Lund-Mackay score.
ΔValue = value (week 32)−value (baseline, week 0).
*$p < 0.05$, **$p < 0.01$ vs. each low groups.

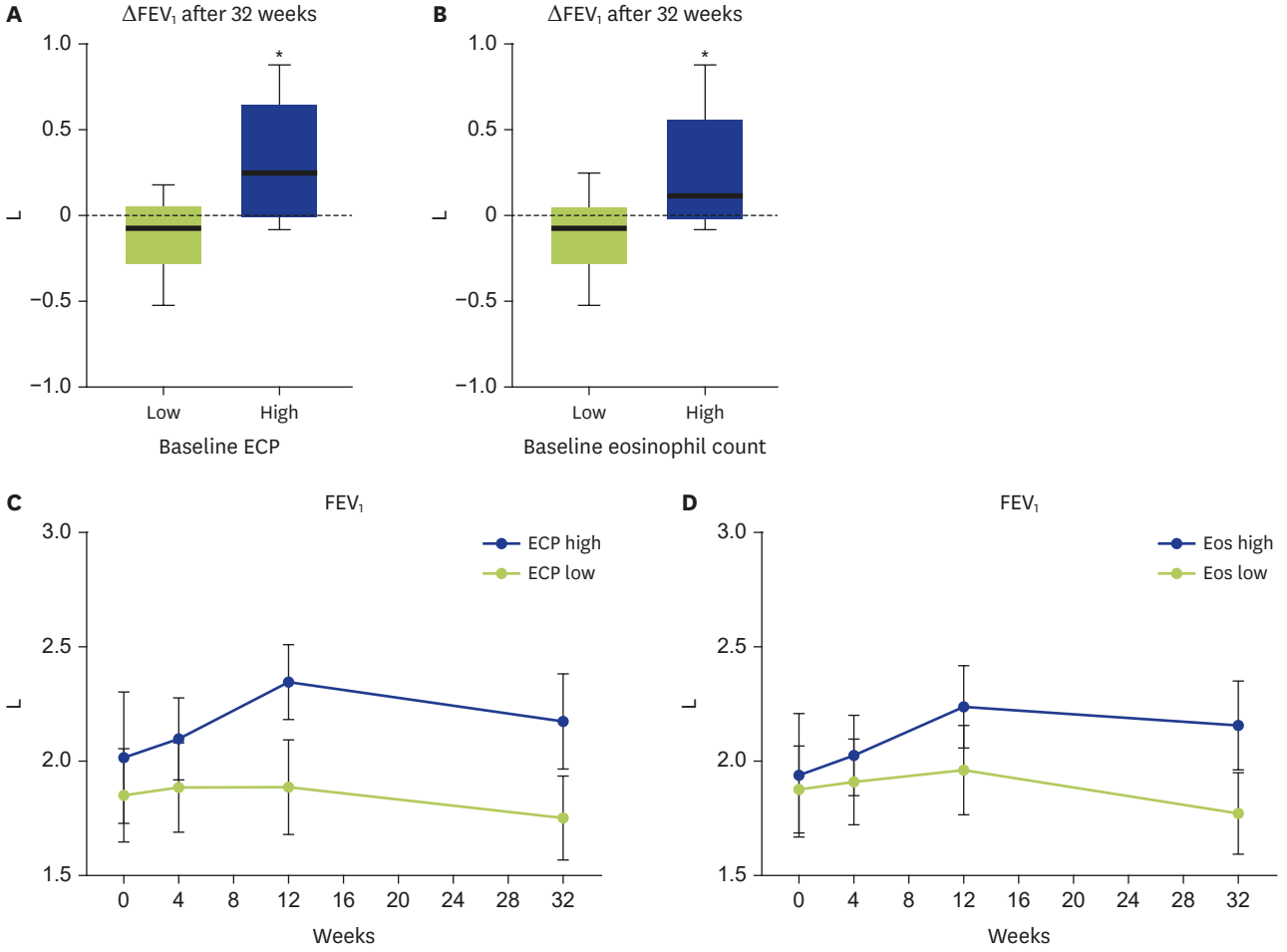

Fig. 3. Relationship between baseline value of biomarkers and increase in forced expiratory volume in 1 second (FEV_1) after 32 weeks treatment. ΔFEV_1 represents the increase in FEV_1 after 32 weeks of treatment by mepolizumab stratified by baseline ECP (12.3 μg/L) (A) or eosinophil counts (403/μL) (B). The boxes represent medians and bars represent minimum to maximum values, *$p < 0.05$ vs. each low group. Time course of FEV_1 stratified by baseline eosinophil cationic protein (ECP; C) or eosinophil counts (D). Values are shown as the mean ± standard error of the mean.

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Table 3. Relationship between the levels of biomarkers at 4 weeks and further improvement in clinical index toward 32 weeks

Variable	ECP (week 4)		Eosinophil count (week 4)	
	Measurable (n = 13)	Unmeasurable (n = 6)	High (n = 10)	Low (n = 10)
ΔACQ-5 (4–32 wk)	−0.37±0.34	−0.48±0.52	−0.56±0.37	−0.22±0.37
ΔAHQ (4–32 wk)	−9.9±6.2	−6.5±8.3	−14.9±6.7	−3.0±6.0
ΔACT (4–32 wk)	2.2±1.2	−0.17±1.58	3.1±1.3	−0.1±1.1
Δ%FEV_1 (%, 4–32 wk)	1.88±3.60*	−14.18±5.34	2.76±5.26	−5.32±4.96
ΔFEV_1 (mL, 4–32 wk)	27.3±61.8*	−256.0±91.7	45.0±95.4	−92.2±89.9

Values are presented as the mean ± standard error of the mean.
ΔValue = value (week 32)−value (week 4).
ECP, eosinophil cationic protein; ACQ, Asthma Control Questionnaire; AHQ, Asthma Health Questionnaire; ACT, Asthma Control Test; %FEV_1, forced expiratory volume in one second (FEV_1); % to predicted value.
*$p < 0.05$ vs. unmeasurable group.

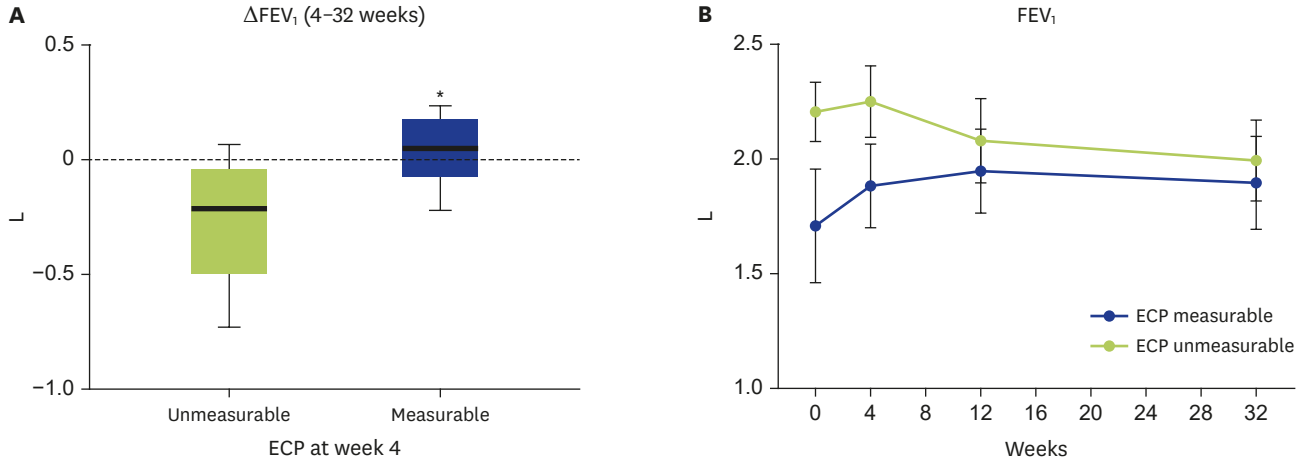

Fig. 4. Relationship between serum eosinophil cationic protein (ECP) levels at 4 weeks after mepolizumab injection and further changes in forced expiratory volume in 1 second (FEV_1). (A) ΔFEV_1 represents the increase in FEV_1 during weeks 4 and 32. Groups are divided by the lowest measurable limit in eosinophil cationic protein (ECP, <2 μg/L) at week 4. The boxes represent medians and bars represent minimum to maximum values, *$p < 0.05$ vs. ECP unmeasurable group. (B) Time course of FEV_1 stratified by ECP levels at week 4. Values are shown as the mean ± standard error of the mean.

As the relationship between baseline ECP level and improvement in FEV_1 was observed, we further investigated if the levels of ECP or eosinophil count after 4 weeks treatment by mepolizumab played a role in the further improvement toward 32 weeks. Our results showed that further improvement in FEV_1 based on eosinophil counts at 4 weeks was not significantly different between groups (**Table 3**). In contrast, serum ECP was still measurable in 12 patients after 4 weeks of treatment and further improvement in FEV_1 toward 32 weeks (FEV_1 at 32 weeks–FEV_1 at 4 weeks) in these patients was significantly higher than those with unmeasurable levels of ECP (**Table 3**, **Fig. 4A**). FEV_1 was further increased toward 12 weeks in patients with measurable levels of ECP only (**Fig. 4B**). In patients with measurable ECP levels at week 4, the frequency of comorbid ECRS (83.3% vs. 16.7%, vs. unmeasurable patients, $p < 0.01$) or nasal polyp (58.3% vs. 0%, $p < 0.05$) and JESREC score (12.4 ± 1.4 vs. 6.0 ± 2.0, $p < 0.01$) was significantly higher and %FEV_1 at baseline (week 0) was significantly lower as compared to ECP unmeasurable group (82.9% ± 7.8% vs. 113.0% ± 10.1%, $p < 0.05$).

DISCUSSION

Our study was the first to show that serum galectin-10 significantly and rapidly decreased 4 weeks after initial injection of mepolizumab as well as the level of ECP by licensed dose,

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100 mg of mepolizumab subcutaneously injected per 4 weeks, and administration route of mepolizumab in real-world settings. Both high ECP and eosinophil count at baseline related to better response in FEV_1 after mepolizumab treatment. Measurable ECP level at 4 weeks after mepolizumab treatment related to the further increase or maintenance of FEV_1 toward week 32, suggesting that the residual ECP activity after early mepolizumab treatment indicates the merit for continuing mepolizumab. In addition, the levels of ECP at baseline were significantly related to the higher prevalence of nasal polyp, JESREC score for diagnosis of ECRS, and LM score, suggesting the close relationship between ECP and nasal comorbidities.

As for time course of biomarkers after mepolizumab treatment, we firstly identified that the level of serum galectin-10 significantly decreased 4 weeks after a single injection of mepolizumab. CLC was firstly identified in cardiac blood and spleen of a patient with leukemia in 1853 and Ernst Viktor von Leyden identified the crystal in the sputum of patients with asthma in 1872 [12]. CLC protein has been assigned to galectin-10 [13], which is a predominant protein within the cytoplasm of eosinophils and the 5th most abundant protein in blood eosinophils [4]. Furthermore, recent evidence suggests that CLCs promote the pathology of asthma [4]. Ueki et al. [14] identified that intracytoplasmic CLCs are formed during the eosinophil extracellular trap cell death (EETosis) followed by the release of CLCs and soluble galectin-10. As EETosis has been evident in various allergic diseases including ECRS [15], the released galectin-10 levels in serum might reflect the activity of eosinophil-related diseases. Although the levels of galectin-10 have been assessed in the tissues from patients with eosinophilic esophagitis [15], or nasal secretion during nonallergic rhinitis of patients with eosinophilia syndrome [16], aspirin-sensitive respiratory disease [17], and chronic rhinosinusitis (CRS) with nasal polyps [18], the clinical relevance of serum galectin-10 is totally unknown to date. In this study, the rapid decrease in serum galection-10 after mepolizumab treatment suggested the resolutions of CLC-mediated inflammation by mepolizumab, warranting further investigation.

In this study, mepolizumab significantly reduced the levels of serum ECP by week 4 and maintained it at a low level until week 32. Although a study not using licensed dose or route of administration [19], and a recent *post hoc* analysis of the study called Mepolizumab as Adjunctive Therapy in Patients with Severe Asthma (MENSA) trial showed the decrease in serum ECP [20], we firstly analyzed the precise time course of ECP levels in real-world settings. In contrast, the levels of basophil count, FeNO, and serum IgE was not significantly affected by mepolizumab treatment. Our findings were consistent with those of a recent study that reported that basophils count was not altered by mepolizumab treatment [21]. We also previously reported that basophils express ~100-fold higher levels of IL-3Rα than IL-5Rα and the rank order of potency for basophil survival was IL-3 > IL-5 = granulocyte macrophage-colony stimulating factor, with IL-3 being 10-fold more potent than the others [22]. In line with above *in vitro* findings, the significant effect of IL-5 blockade by mepolizumab on basophils count was not observed, suggesting that a mediator other than IL-5 maintains the blood basophil count. We also confirmed the results from previous studies showing that no significant changes in the level of FeNO [23, 24] or IgE [25]. NO is produced by inducible NO synthase, whose expression is regulated by IL-4 and IL-13 [26]. In addition, the levels of FeNO and serum IgE were down-regulated after treatment by IL-4Rα antibody dupilumab [27], suggesting that those molecules are predominantly regulated by IL-4 and IL-13.

As this study was performed in real-world settings, patients with smoking history of more than 10-pack years or without airway reversibility were recruited. Nevertheless, the significant

improvements in ACT and ACQ-5 scores were observed in line with recent real-world study [28]. The mean eosinophil count was 572/μL in this study and 290/μL in the phase 3 MENSA trial [2], suggesting that highly eosinophilic patients were eligible for mepolizumab treatment in real-world settings, thus obtaining the apparent response to mepolizumab.

Regarding FEV_1, although the MENSA study showed the significant improvement [2], our study did not show significant improvement in overall analysis possibly partly due to the difference in the baseline characteristics of patients. In this study, recruited patients had low reversibility and high baseline FEV_1; the mean reversibility in FEV_1 was 27.9% and 9.3%, and baseline FEV_1, % predicted was 59.3% and 88% in MENSA study and current study, respectively. Nevertheless, the response of FEV_1 to mepolizumab was heterogenous. The increase in FEV_1 after 32 weeks treatment in patients with high value of ECP or eosinophil count was significantly higher as compared to patients with low value (**Table 2**; **Fig. 3A, B**). Interestingly, a previous cluster analysis identified 2 clusters: a cluster with significantly higher serum ECP levels, low FEV_1 values, and good FEV_1 improvement in response to asthma therapy and another cluster with low ECP serum levels, higher FEV_1 values, and no FEV_1 improvement in response to asthma therapy [29]. The absence of significant difference in blood eosinophil counts between 2 clusters demonstrates the relative importance of the relationship between serum ECP and better treatment response in FEV_1. Furthermore, another study showed that the level of ECP was better correlated with low FEV_1 than eosinophil count [30], suggesting that ECP could be a sensitive marker of airflow obstruction in asthma. The difference in the effects of eosinophil count and serum ECP on FEV_1 may be because serum ECP reflects the functional releasability of eosinophils *in vivo*. In addition, regarding the method for measuring serum ECP in laboratory testing, eosinophils continue to degranulate ECP after blood drawal in whole blood when incubated at 37°C [31]. Although the serum was collected after leaving whole blood samples at room temperature in this study, measured ECP levels may to some extent include degranulated ECP during incubation *in vitro*. Taken together, the level of ECP may partly reflect the functional activity of eosinophils, in addition to the mere number of eosinophils.

In the current study, mepolizumab significantly improved LM scores in patients with severe asthma, which is consistent with previous reports on ECRS in patients with asthma [32]. Although the improvement in LM score tended to be higher in high ECP group ($p = 0.063$), no significant relationship with eosinophil count was observed. The patients with ECRS or CRS with nasal polyps have been shown to have a significantly higher level of ECP in nasal tissue than patients with non-eosinophilic CRS and control patients [33, 34]. In addition, serum ECP may be a feasible predictor for early postoperative recurrence of nasal polyps [35]. That evidence suggested that ECRS pathophysiology does not depend on the mere presence of eosinophil but on the degranulation of eosinophils. Our finding indicates the usefulness of serum ECP over blood eosinophil count as potential predictive factor of mepolizumab efficacy for ECRS in severe asthma. Galectin-10 in nasal secretion has been shown to be significantly higher in patients with recurrent nasal polyp [18], suggesting an important role of galectin-10 in formation of nasal polyp. However, the clinical relevance of serum galectin-10 in nasal polyp or ECRS has not been reported yet. The relationship between serum galectin-10 and prevalence of ECRS was not shown in this study. However, ECP and galectin-10 are stored in different part of eosinophils, i.e., granules or cytoplasm, and levels of those molecules were not significantly correlated, suggesting different pathological roles in eosinophilic diseases. Thus, further investigation concerning the serum galectin-10 as a potential biomarker for nasal diseases is required.

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Predictive factors for the response to mepolizumab treatment have been mainly analyzed concerning exacerbations as outcomes and there have been few reports for prediction factor for improvement in FEV_1. Combined *post hoc* analysis of DREAM and MENSA study showed the weak relationship between eosinophil count >500/μL and improvement in FEV_1 [7]. However, the role of ECP for prediction of FEV_1 response has not been reported. In our study, patients with high ECP showed the significantly better response in FEV_1 after mepolizumab treatment for 32 weeks in real-world settings, suggesting ECP as a suitable predictive marker for FEV_1 response as compared to eosinophils. During our analysis, a *post hoc* analysis of MENSA study showed that although ECP did not distinguish the responder concerning exacerbation, patients with high ECP level showed numerically better response to mepolizumab for FEV_1 [20].

Continuation rule after starting mepolizumab treatment has not been established yet. Although rules-based blood eosinophils, physician-rated response to treatment, FEV_1, ACQ-5 score, and exacerbation reduction were assessed at week 16, no reliable continuation rule was established [36]. However, the relationship between the levels of biomarker during treatment and further response has not been investigated. In our current study, patients with measurable ECP after 4 weeks treatment showed the further improvement in FEV_1 toward 32 weeks (**Table 3**, **Fig. 4A**). Our results suggested that measuring the ECP level during treatment as an eligible biomarker for the continuation rule and the residual ECP during treatment might be a trait to be further treated during mepolizumab treatment. Further investigation with a greater number of patients is needed.

As limitations of current study, the establishment of biomarkers as predictive factors for the efficacy requires further analysis with a larger number of patients in the future. Nevertheless, our analysis of the time course of clinical index or biomarkers showed high *p* value, suggesting the firm results.

In conclusion, we showed that the levels of serum galectin-10 and ECP rapidly decreased 4 weeks after initial injection of mepolizumab. The significant relationship between serum ECP and nasal comorbidities or better response to mepolizumab in FEV_1 suggested the prominent role of serum ECP over eosinophil count as a biomarker for patients with severe asthma treated by mepolizumab.

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