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ORIGINAL RESEARCH

Evaluation of the Correlation Between Nasal Secretion ECP-MPO Test Papers and Immune Markers in Subcutaneous Immunotherapy of Dust Mites

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Purpose: Up to now, there is no generally accepted biomarker to indicate the clinical response of immunotherapy. This study mainly analyzed the correlation between eosinophil cationic protein-myeloperoxidase (ECP-MPO) test papers and other immunotherapy indices in subcutaneous immunotherapy of dust mites and to explore whether the test paper can be used as an auxiliary index to quickly evaluate the efficacy of immunotherapy.

Patients and methods: This study included 53 participants who received subcutaneous immunotherapy at the allergy clinic of Renmin Hospital of Wuhan University and 28 control participants. Six visits were conducted during a prospective study over one year. The results of the ECP-MPO test paper, nasal secretion eosinophil smear and count, nasal secretion ECP concentration, and clinical symptom scores were collected during five follow-up visits after the start of subcutaneous immunotherapy. Th1/Th2/Th17 cytokines, chemokines, IgE, IgG4 against dust mite components, and ECP concentrations were detected in the serum of participants at baseline, six months, and one year after subcutaneous immunotherapy.

Results: The ECP test paper is not only easy to operate, but also can effectively and quickly detect the concentrations of ECP in the nasal secretion and diagnose allergic rhinitis. Symptom score is an important index for evaluating clinical immune efficacy, during subcutaneous immunotherapy, the ECP test paper showed a positive correlation with the symptom score. Simultaneously, during immunotherapy, the changes in the chromogenic grading of the test paper were synchronized with the changes in inflammatory cytokines and eosinophilic chemokines in Th2 cells of serum dust mite IgE. The sIgG4 against dust mites weakly negatively correlated with the concentration of ECP in nasal secretions and the color classification of the ECP test paper.

Conclusion: The ECP-MPO test paper has a certain correlation with subcutaneous immunotherapy markers of allergic rhinitis, indicating that the ECP test paper may become an auxiliary biomarker to replace other complex laboratory tests.

Keywords: dust mites, ECP-MPO test paper, subcutaneous immunotherapy, allergic rhinitis

Introduction

Patients with rhinitis are divided into four main subgroups: allergic rhinitis (AR), infectious rhinitis, nonallergic noninfectious rhinitis, and mixed rhinitis. AR is the most common noninfectious disease and is defined as symptomatic nasal inflammation caused by the inhalation of allergens in allergic individuals.[1] Rhinitis not only creates substantial direct medical expenses for patients, but is also an important reason for reduction in work and school time, as well as a decline in work efficiency and academic performance.[2] Eosinophils increase in atopic diseases such as AR and asthma.

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Eosinophil cationic protein (ECP) is recognized as a marker of eosinophils and can be quantified in serum, bronchoalveolar lavage fluid, and nasal secretions.[3] Some studies have shown that the measurement of ECP levels can effectively monitor the disease activity of AR and the efficacy of therapeutic drugs.[4]

At present, the treatment of AR mainly includes drug therapy, allergen immunotherapy (AIT) and surgery. For patients with AR who do not respond to conventional drug therapy, AIT is currently the only option for symptom relief.[5] AIT reduces symptom scores and drug demand, thereby improving quality of life, changing the course of allergic disease, and providing long-term clinical benefits after discontinuation of treatment. The gold standard for evaluating the efficacy of AIT is the assessment of clinical symptoms and drug scores during natural allergen exposure.[6] Biomarkers of AIT play a core role in personalized medicine. Several pollen AIT studies have reported that specific IgE (sIgE) levels briefly increase and then decrease after treatment.[7] The ratio of sIgE to total IgE (sIgE/tIgE) is a promising predictive marker.[8] Studies have shown that sIgG4 levels increase in a time-dependent manner during treatment, and sIgG4 is considered to be a blocking antibody that blocks the activation and degranulation of effector cells.[9] In addition, a hypothetical mechanism for long-term clinical tolerance after AIT involves the transition from a Th2 response to a Th1 response.[10] However, to date, there are no validated or generally accepted biomarkers that can predict or indicate the clinical response to AIT. Moreover, the detection of IgE, IgG4, and cytokines depends on laboratory testing over a relatively long period. Therefore, it is particularly important to develop a rapid and convenient detection method to evaluate the efficacy of AIT.

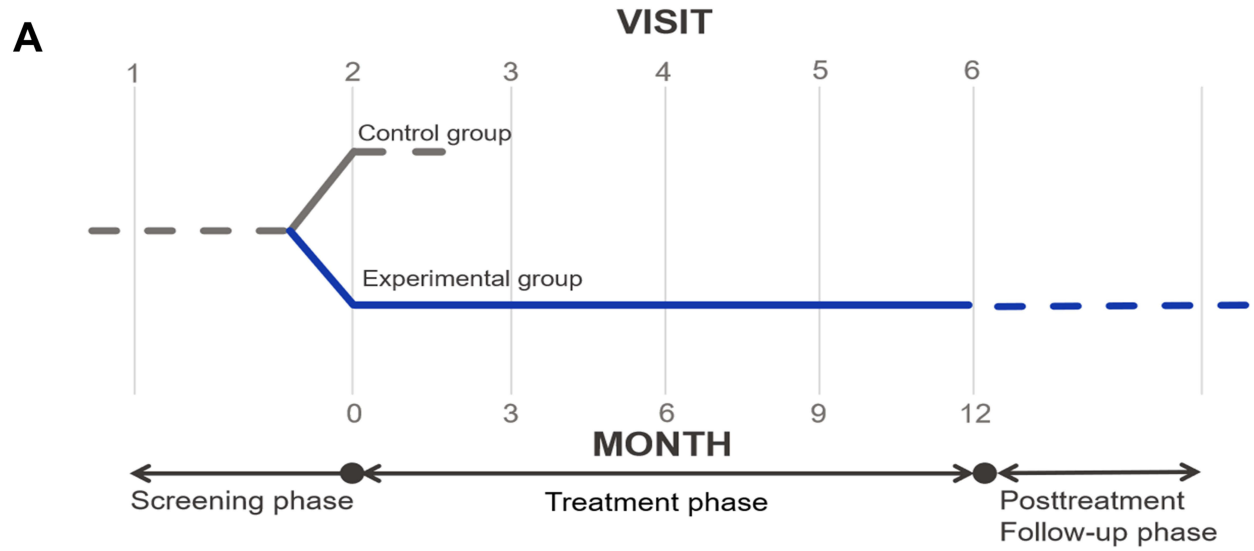
Studies have shown that nasal nitric oxide is significantly reduced in patients with perennial allergic rhinitis after AIT.[11] Studies have also shown that AIT can reduce myeloperoxidase levels in nasal neutrophils of allergic patients.[12] Cypress allergy immunotherapy can significantly reduce ECP levels in nasal lavage solution.[13] It can be seen that the changes of nasal biomarkers in allergic patients are closely related to the efficacy of AIT, and nasal biomarkers have shown potential clinical efficacy evaluation ability. The ECP-MPO real-time test paper used in this study can detect ECP and MPO in patient nasal secretions within 10 min, making it possible to distinguish AR from infectious rhinitis in a short time. Because of the design of the experiment, the function of ECP test paper is only discussed in this paper. In a previous study, we evaluated the diagnostic value of ECP-MPO real-time test papers before and after drug treatment for AR.[14] This study aimed to explore the function of the ECP-MPO real-time test paper in evaluating and monitoring the efficacy of subcutaneous immunotherapy in patients with AR using a comprehensive comparative analysis with biomarkers of efficacy, so as to further discuss the possibility of ECP test paper as an immune marker of AIT.

Methods

Study Process

In this study, patients with AR and control participants were recruited to explore the application value of ECP test holes in subcutaneous immunotherapy. This was a 1-year, long-term, prospective, case-control study.

The study consisted of the participants attending six visits. The first visit (V1) was a screening period of 1–8 days, which mainly included screening patients, obtaining informed consent, and collecting basic information (including race, height, weight, history of smoking and drinking, and other allergic diseases). The second visit (V2) was to initiate subcutaneous immunotherapy. All patients who received subcutaneous immunotherapy followed the manufacturer's instructions using an NHD [NovoHelisen Depot; Allergopharma, Reinbek, Germany]. NHD is an allergen extract derived from *Dermatophagoides pteronyssinus* (Der p) and *Dermatophagoides farinae* (Der f). The manufacturers claim that the major dust mite allergen contents (Der p and Der f) are 6.4μg/mL for NHD dust mite. Patients received injections weekly at a volume of 0.2, 0.4, and 0.8 mL in the No. 1 to 2 vials and 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mL in the No. 3 vial, reaching the maintenance dose, 5000 Treatment Units. Then, the maintenance dose was given on a 4-week basis in both groups. The patients' electronic symptom questionnaires were collected, serum analyzed, and nasal secretion smears tested using ECP-MPO test papers. The third to sixth visits (V3–V6) were conducted at 3, 6, 9, and 12 months after subcutaneous immunotherapy, and the electronic symptom questionnaire and nasal secretion smears were collected again. Serum samples were collected at V2, V4, and V6 (Figure 1A). At the end of the trial, patients continued the next step of immunotherapy.



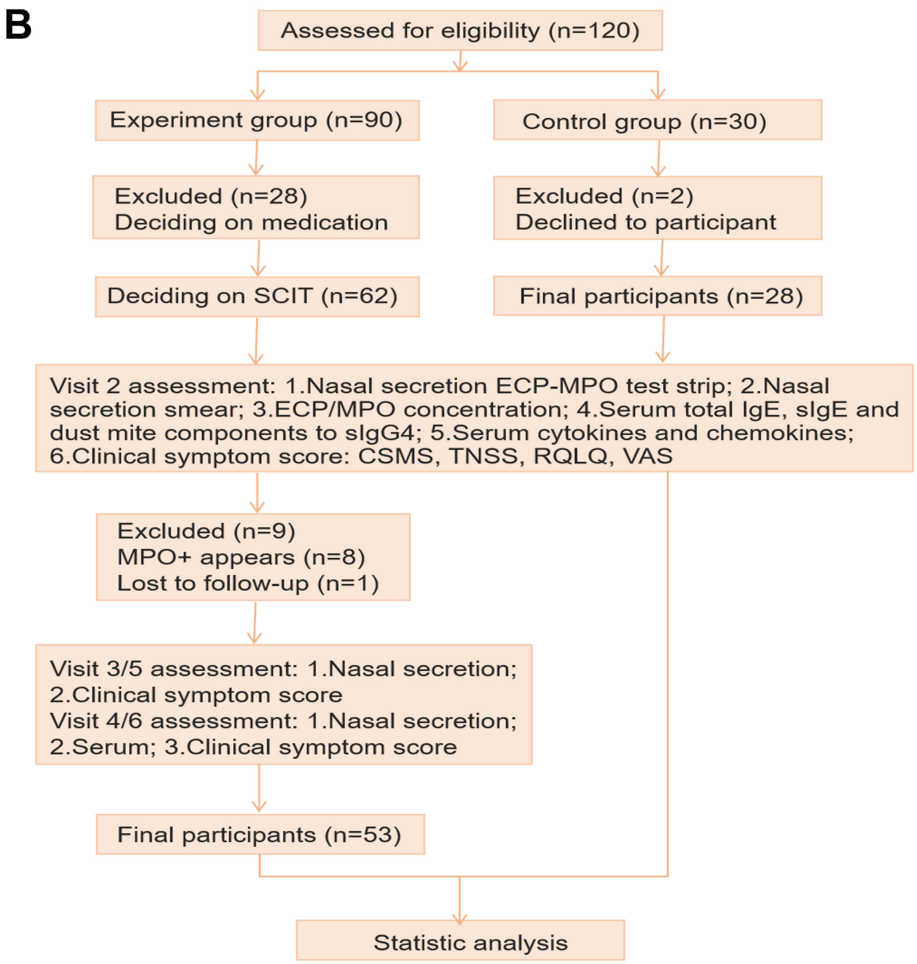


Figure 1 Study design and participants disposition. (**A**) Study design of the subcutaneous immunotherapy experimental and control groups. (**B**) Subcutaneous immunotherapy experimental and control group participants disposition.

Abbreviation: SCIT, subcutaneous injection; CSMS, combined symptom and medication score; TNSS, total nasal symptom score; RQLQ, rhinitis quality of life questionnaire; VAS, visual analog scale; ECP, eosinophil cationic protein; MPO, Myeloperoxidase.

Study Participants

The study participants were outpatients at Renmin Hospital of Wuhan University. Patients meeting the inclusion criteria were recruited. All participants provided signed informed consent and the research program was approved by the Ethics Committee of the Renmin Hospital of Wuhan University (WDRY2021-K052). This study was conducted in accordance with the Declaration of Helsinki. The study included a total of 120 participants. Among them were 90 patients with AR, 62 of whom received subcutaneous immunotherapy. Nine with infection or lost to follow-up were excluded. Therefore, 53 participants were included in the trial group. There were 30 people in the control group, and two people who refused to participate were excluded. Finally, 28 control participants were included in the statistical analyses (Figure 1B). The main inclusion criteria for AR in this trial were identical to those used in our previous study.[14] Patients with nasal mucosal damage or severe chronic disease (severe liver disease, kidney disease, heart disease or blood disease, etc.) were excluded.

Symptom Assessment Questionnaire

When the participants were interviewed during V2–V6, their clinical symptoms were assessed. The assessment asked the participants to fill in an electronic questionnaire, including the comprehensive symptom and medication score (CSMS), total score of rhinitis symptoms (TNSS), rhinitis quality of life questionnaire (RQLQ), and visual analog scale (VAS).

Smear and Cell Count of Nasal Secretions

The nasal secretions of the patients were evenly and thinly coated on slides, naturally air-dried, stained with Swiss-Giemsa, counted, and classified under a light microscope after staining was completed. The number of eosinophils or neutrophils was observed under a microscope at 400 × magnification and the average value of five visual fields was recorded.

Detection of Serum

The serum of the patients of the study was collected and serum levels of IL-2, IL-4, IL-6, IFN- γ, TNF- α, IL-17A, IL-10, CCL17, CCL22, CCL11 and CCL26 were detected using an ELISA detection kit (Bioswamp, Wuhan, China). Experiments were performed in strict accordance with the manufacturer's instructions. Protein chip was used to detect the component-specific IgG4 of dust mites, including Derp-1, Derf-1, Derp-2, Derf-2, Derp-5, Derp-7, Derp-10, Derp-21, Derp-23.

ECP-MPO Test Paper

An ECP-MPO test kit (Dabai Xiaobai Technology Co., Wuhan, China) was used. The nasal secretions of the participants were collected using the filter paper in the test box. After the filter paper fully absorbed the nasal secretions, the surface residue was gently wiped off, the 0.5 cm × 0.4 cm filter paper strip cut off in the 1 mL sample extract, and another 0.5 cm × 0.4 cm filter paper strip cut off in the 5 mL 1% sample extract. After shaking on an oscillator for 15s, the sample extraction liquid was dropped into the sample hole of the test paper card and left for 10 min before reading the results.[14]

Sample Size Calculation and Statistical Analysis

For this study, it was assumed that ECP-MPO test papers could effectively distinguish between AR and control patients. The sensitivity and specificity of the ECP-MPO test were assumed to be greater than 0.6. In a preliminary experiment, the sensitivity and specificity of the test paper for AR diagnosis were 0.9400 and 0.86961, respectively. When $\alpha = 0.05$ (unilateral), $\beta = 0.1$ and the ratio of the test group to the control group was 2:1, the sample size was estimated using PASS11 software (NCSS, Kaysville, Utah, USA). The results showed that at least 73 participants were required to be included, including 49 in the test group and 24 in the control group. Considering a loss to follow-up rate of 20%, the study included 62 patients in the experimental group and 30 patients in the control group.[15,16]

Using SPSS 19.0 (IBM, Armonk, NY, USA) and R 4.0.3 (R Foundation, Vienna, Austria), descriptive analysis, normal distribution and analysis of variance were used for statistical analysis. Normally distributed data are expressed as mean ± standard deviation. The Shapiro–Wilk test was used to test normality. One-way analysis of variance was used for

each group, and Tukey's test was used for pairwise comparisons. Statistical significance was set at $P < 0.05$. All correlations were tested using the Pearson correlation test, and the kappa test was used to verify the consistency between the diagnosis and the gold standard diagnosis.

Results

Study Participants

This study included 53 participants who received subcutaneous immunotherapy and 28 control participants at the allergy clinic of Renmin Hospital of Wuhan University. There were 26 males and 27 females in the test group, and 15 males and 13 females in the control group. The average age of the experimental group was 20.44 years old, average height 154.31 cm, average weight 61.10 kg, 3% had a history of smoking, 4% had a history of drinking, and 4% had other allergic diseases. The average age of the control group was 21.68 years old, average height 151.04 cm, average weight 48.21 kg, 1% had a history of smoking, 2% had a history of drinking, and no participants had other allergic diseases. (Table 1).

ECP Test Paper Can Effectively Diagnose Allergic Rhinitis

Based on the color contrast between the contrast line and the test line, we divided the test paper results into level 0, 1, 2, and 3. Level 3, the detection and control line colour intensities are similar; level 2, the detection line is slightly lighter than the control line; level 1, the detection line is only slightly coloured; grade 0, the detection line is not coloured (Figure 2A). The distribution of eosinophils in different grades of test paper is shown in Figure 2B. The color grading of the ECP test paper was positively correlated with the concentration of ECP in nasal secretions ($r=0.875$, $P<0.001$, Figure 2C); with an increase in eosinophils in nasal secretions, the degree of color of the ECP test paper also increased ($r=0.856$, $P<0.001$, Figure 2D).

Table 1 Baseline Characteristics of Trial and Control Participant

Characteristic	Trial participant (n=53)	Control participant (n=28)
Gender		
Men	26 (49.1)	15 (53.6)
Women	27 (50.9)	13 (46.4)
Age (y)		
Mean ± SD	20.44±13.69	21.68±16.27
Median	15	12.5
Height (cm)		
Mean ± SD	154.31±21.49	151.04±19.69
Median	159.00	158.50
Weight (kg)		
Mean ± SD	61.10±36.33	48.21±18.47
Median	57.00	51.00
Smoke, n (%)		
Yes	3 (5.7)	1 (3.6)
No	50 (94.3)	27 (96.4)
Drink alcohol, n (%)		
Yes	4 (7.5)	2 (7.6)
No	49 (92.5)	26 (92.9)
Other allergic diseases, n (%)		
Yes	4 (7.5)	0
No	49 (92.5)	28 (100)

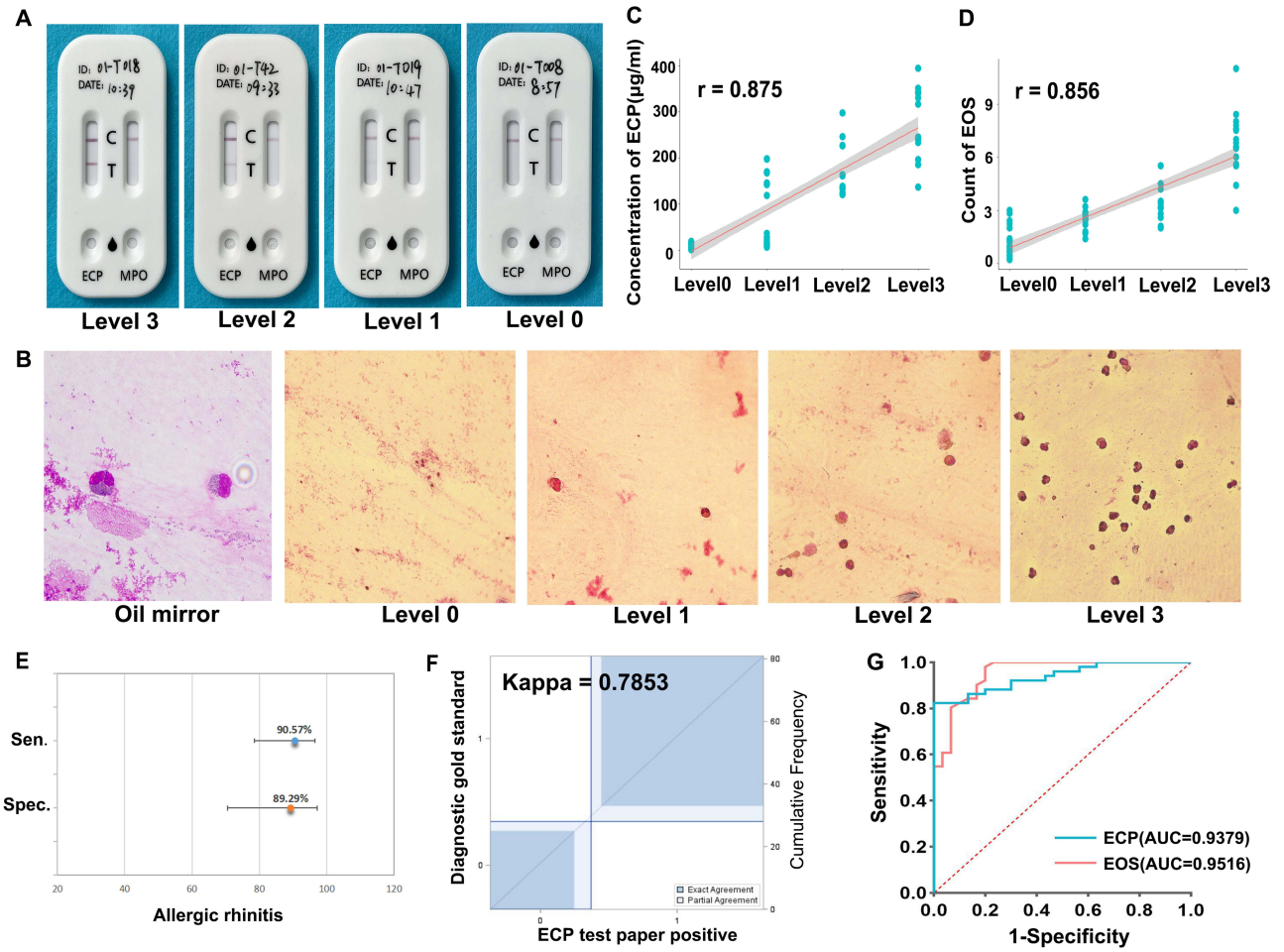


Figure 2 ECP test paper can effectively diagnose allergic rhinitis. (**A**) The color grading of the ECP test paper results. (**B**) Microscopic examination of eosinophils in nasal secretions of different levels of participants. (**C**) Correlation between the color grade of the ECP test paper and the ECP concentrations in nasal secretions. (**D**) Correlation between the color grade of the ECP test paper and count of eosinophils in nasal secretions. (**E**) Sensitivity and specificity of ECP test paper diagnosis. (**F**) Kappa test of positive ECP test paper results and clinical diagnosis of allergic rhinitis. (**G**) The receiver operating characteristic (ROC) curve of ECP concentration and eosinophil count in nasal secretions used to evaluate the diagnostic performance when the ECP test paper was positive.
Abbreviation: ECP, Eosinophil cationic protein; Sen, Sensitivity; Spec, Specificity; AUC, Area under curve.

For the clinical gold standard of AR diagnosis, the sensitivity of the ECP test paper is 90.57% (95% confidence interval (CI) 78.58–96.47%) and the specificity is 89.29% (95% CI 70.63–97.19%) (Figure 2E). Using the kappa diagnostic consistency test, we found that the diagnoses of AR with the ECP test paper were consistent with the gold standard for clinical diagnosis, with kappa values of 0.7853 (Figure 2F). In addition, the ECP concentration in nasal secretions and receiver operating characteristic (ROC) curve of eosinophils were used to evaluate the diagnostic performance of the positive ECP test paper. The areas under the curve (AUC) of the two were 0.9379 and 0.9516, respectively. When the ECP test paper was positive, the cutoff values of the two were 19.31 μg/mL and 1.55 per high power visual field, respectively (Figure 2G).

Changes of Test Paper and Clinical Symptoms During Subcutaneous Immunotherapy

During the subcutaneous immunization, the color of the ECP test paper became increasingly lighter (Figure 3A). The ROC curve of treatment time was used to calculate the negative time of the ECP test paper. The AUC of the treatment time was 0.7422, and during immunotherapy, when the ECP test result turned negative, the cutoff value for the treatment time was 4.5 months (Figure 3B). Similarly, with the passage of time of subcutaneous immunotherapy, the concentration of ECP in the nasal secretions and serum also decreased gradually; however, the decrease in serum ECP concentration was not statistically significant. The concentration of ECP in local nasal secretions decreased significantly in the third

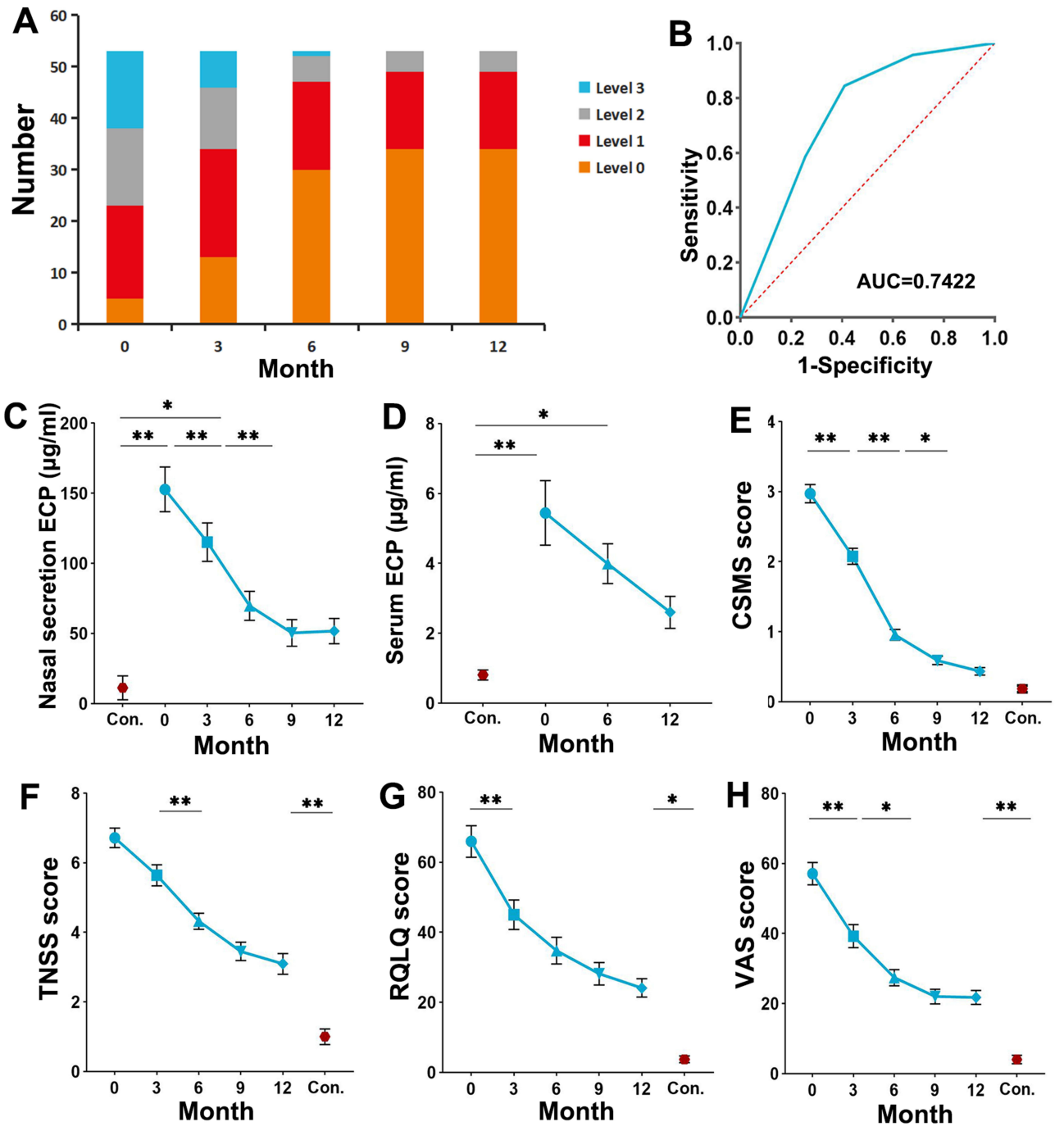


Figure 3 Changes of test paper and clinical symptoms. (**A**) Clinical changes of the color grade of the ECP test paper during immunotherapy. (**B**) The receiver operating characteristic (ROC) curve of immunotherapy time used to evaluate the diagnostic function of the ECP test paper turning negative. (**C–H**) Clinical changes during immunotherapy (concentration of nasal secretion ECP, concentration of serum ECP, CSMS, TNSS, RQLQ, and VAS). *, P<0.05; **, P<0.01.

Abbreviation: ECP, Eosinophil cationic protein; Con, Control group; AUC, Area under curve; CSMS, combined symptom and medication; TNSS, Total nasal symptom score; RQLQ, Rhinitis quality of life questionnaire; VAS, Visual analog scale.

month compared with the baseline period and further decreased in the sixth month (Figure 3C and D). Based on the statistical analysis of the symptom scores of the subjects during immunotherapy, we found that the CSMS, TNSS, RQLQ and VAS scores decreased significantly after 6 months of treatment, and the changes were more stable at 6–12 months (Figure 3E–H).

Correlation Analysis of Test Paper and Clinical Symptoms During Subcutaneous Immunotherapy

We analyzed the correlation between systemic and local ECP concentrations and clinical symptoms and found that there was a positive correlation between nasal secretion of ECP and clinical symptom score at 0, 3, 6, 9 and 12 months, with the correlation coefficient was between 0.3 and 0.7, and the P-value was between <0.001 and 0.015. The detailed correlation coefficients are shown in Figure 4A–D. However, the correlation between serum ECP levels and clinical symptoms was weak, and the correlation coefficient is between 0.02 and 0.4, and the P-value was between 0.005 and 0.866 (Figure 4E–H). Furthermore, the test paper grades of the three desensitization injection nodes were analyzed using CSMS, TNSS, RQLQ, and VAS scores. It was also found that the test paper grades were positively correlated with clinical symptom scores, with a correlation coefficient of approximately 0.6, and the P-value was <0.001(Figure 4I–L).

In addition, we analyzed the correlation among baseline nasal secretion, serum ECP concentration, color grade of the test paper, and clinical symptom score after 12 months of immunotherapy to evaluate the prognostic function of the test paper for immunotherapy. We found no correlation between the baseline test paper classification and symptoms after one year of immunotherapy, the highest correlation coefficient is only 0.305. (Figure 5).

Changes and Correlation Analysis of Serum IgE Antibodies During Subcutaneous Immunotherapy

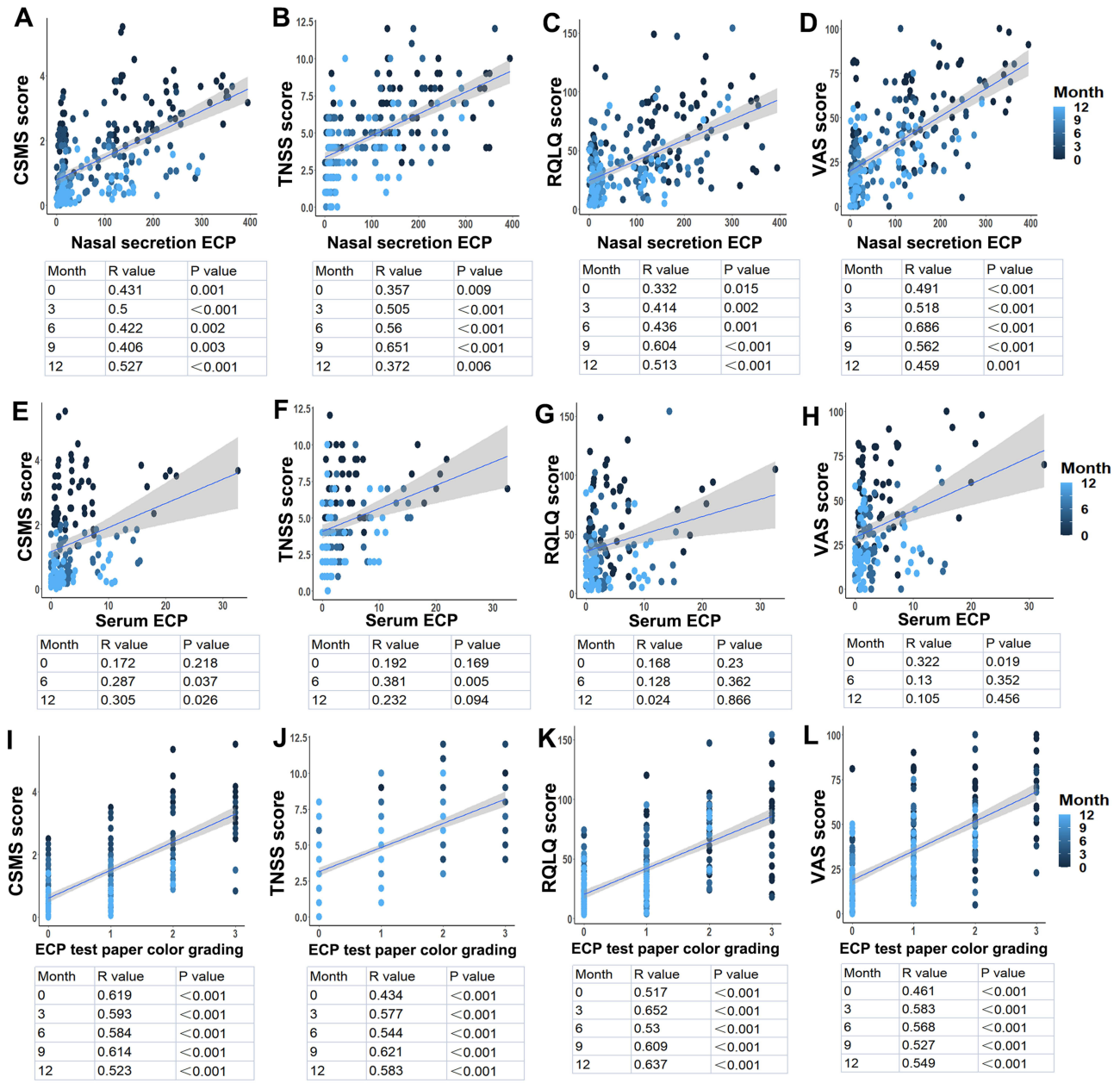
Figure 6 shows the changes in sIgE and total (t)IgE levels during subcutaneous immunotherapy and their correlation with the color grading of the test paper. We found that Der p-IgE, Der f-IgE, and tIgE decreased during subcutaneous immunotherapy, and decreased significantly 6 months before treatment, whereas Der p-IgE/tIgE and Der f-IgE/tIgE levels did not change significantly during subcutaneous immunotherapy. After 12 months of treatment, the levels of Der p-IgE, Der f-IgE, total IgE, Der p-IgE/tIgE and Der f-IgE/tIgE levels in the test group were higher than those in the control group (Figure 6A-E). The concentration of ECP in nasal secretions were positively correlated with Der p-IgE and Der f-IgE (r=0.344~0.619, P=<0.001~0.012, Figure 6F-G), but weakly positively correlated with tIgE, Der p-IgE/tIgE, and Der f-IgE/tIgE (r=0.089~0.433, P=0.001~0.524, Figure 6H-J). In addition, The color grade of the ECP test paper were positively correlated with Der p-IgE and Der f-IgE (r=0.5~0.644, P=<0.001, Figure 6K-L), but weakly positively correlated with tIgE, Der p-IgE/tIgE, and Der f-IgE/tIgE (r=0.155~0.478, P=<0.001~0.268, Figure 6M-O).

Changes and Correlation Analysis of Serum IgG4 Antibodies During Subcutaneous Immunotherapy

Figure 7A shows the concentrations of the dust mite component sIgG4 in the control and experimental groups in baseline, sixth, and twelfth month. We found that, compared with the sixth month, the serum concentrations of Der p1-sIgG4, Der p2-sIgG4, Der p7-sIgG4, Der p21-sIgG4, and Der f2-sIgG4 in the 12th month showed an upward trend, whereas the other components (Der p5-sIgG4, Der p10-sIgG4, Der p23-sIgG4, and Der f1-sIgG4) showed no significant change before and after treatment (Figure 7B and C). Figures 7D–I show that the sIgG4 of dust mites was weakly negatively correlated with the concentration of ECP in nasal secretions and the color classification of the ECP test paper (r=−0.385~-0.009, P=0.004~0.947). The red circle shows that the correlation coefficient is −0.2, which shows that there is a correlation between them outside the circle. The detailed correlation coefficients are shown in Supplementary Table 1.

Changes and Correlation Analysis of Serum Cytokines and Chemokines During Subcutaneous Immunotherapy

Compared with those before immunotherapy, the serum levels of IL-4, IL-6 and IL-17A decreased, while the levels of IL-10, IL-2, IFN- γ and TNF- α increased after treatment (Figure 8A). The levels of IL-4, IL-6 and IL-10 were positively correlated with the color grading of ECP-MPO paper (r=0.324~0.624, P=<0.001~0.017), while the level of IFN-γ was negatively correlated with the chromogenic grade of ECP-MPO paper (r=−0.577~-0.439, P=<0.001~0.001). There was no correlation between the levels of IL-2, TNF- α and IL-17A (r=−0.389~0.219, P=0.004~0.353, Figure 8B). After



Month	R value	P value
0	0.431	0.001
3	0.5	<0.001
6	0.422	0.002
9	0.406	0.003
12	0.527	<0.001

Month	R value	P value
0	0.357	0.009
3	0.505	<0.001
6	0.56	<0.001
9	0.651	<0.001
12	0.372	0.006

Month	R value	P value
0	0.332	0.015
3	0.414	0.002
6	0.436	0.001
9	0.604	<0.001
12	0.513	<0.001

Month	R value	P value
0	0.491	<0.001
3	0.518	<0.001
6	0.686	<0.001
9	0.562	<0.001
12	0.459	0.001

Month	R value	P value
0	0.172	0.218
6	0.287	0.037
12	0.305	0.026

Month	R value	P value
0	0.192	0.169
6	0.381	0.005
12	0.232	0.094

Month	R value	P value
0	0.168	0.23
6	0.128	0.362
12	0.024	0.866

Month	R value	P value
0	0.322	0.019
6	0.13	0.352
12	0.105	0.456

Month	R value	P value
0	0.619	<0.001
3	0.593	<0.001
6	0.584	<0.001
9	0.614	<0.001
12	0.523	<0.001

Month	R value	P value
0	0.434	<0.001
3	0.577	<0.001
6	0.544	<0.001
9	0.621	<0.001
12	0.583	<0.001

Month	R value	P value
0	0.517	<0.001
3	0.652	<0.001
6	0.53	<0.001
9	0.609	<0.001
12	0.637	<0.001

Month	R value	P value
0	0.461	<0.001
3	0.583	<0.001
6	0.568	<0.001
9	0.527	<0.001
12	0.549	<0.001

Figure 4 Correlation analysis of test paper and clinical symptoms. (**A-D**) Correlation between the concentration of nasal secretion ECP and clinical symptom score. (**E-H**) Correlation between the concentration of serum ECP and clinical symptom score. (**I-L**) Correlation between the color grading of the ECP test paper and clinical symptom score.

Abbreviation: ECP, Eosinophil cationic protein; CSMS, combined symptom and medication; TNSS, Total nasal symptom score; RQLQ, Rhinitis quality of life questionnaire; VAS, Visual analog scale.

immunotherapy, CCL17, CCL22, CCL11, and CCL26 showed a downward trend (Figure 8C), and all showed a positive correlation with the color grade of the test paper to varying degrees, among which CCL26 had the best correlation with the color grade of the test paper (r=0.356~0.795, P=<0.001~0.009, Figure 8D). The detailed correlation coefficients are shown in Supplementary Table 2.

Discussion

The plasma concentration of ECP, a marker of eosinophil activation, can be used to detect type 2 inflammation.[17] Currently, ECP are specific indicators for the differential diagnosis of allergy. Allergen immunotherapy is considered the

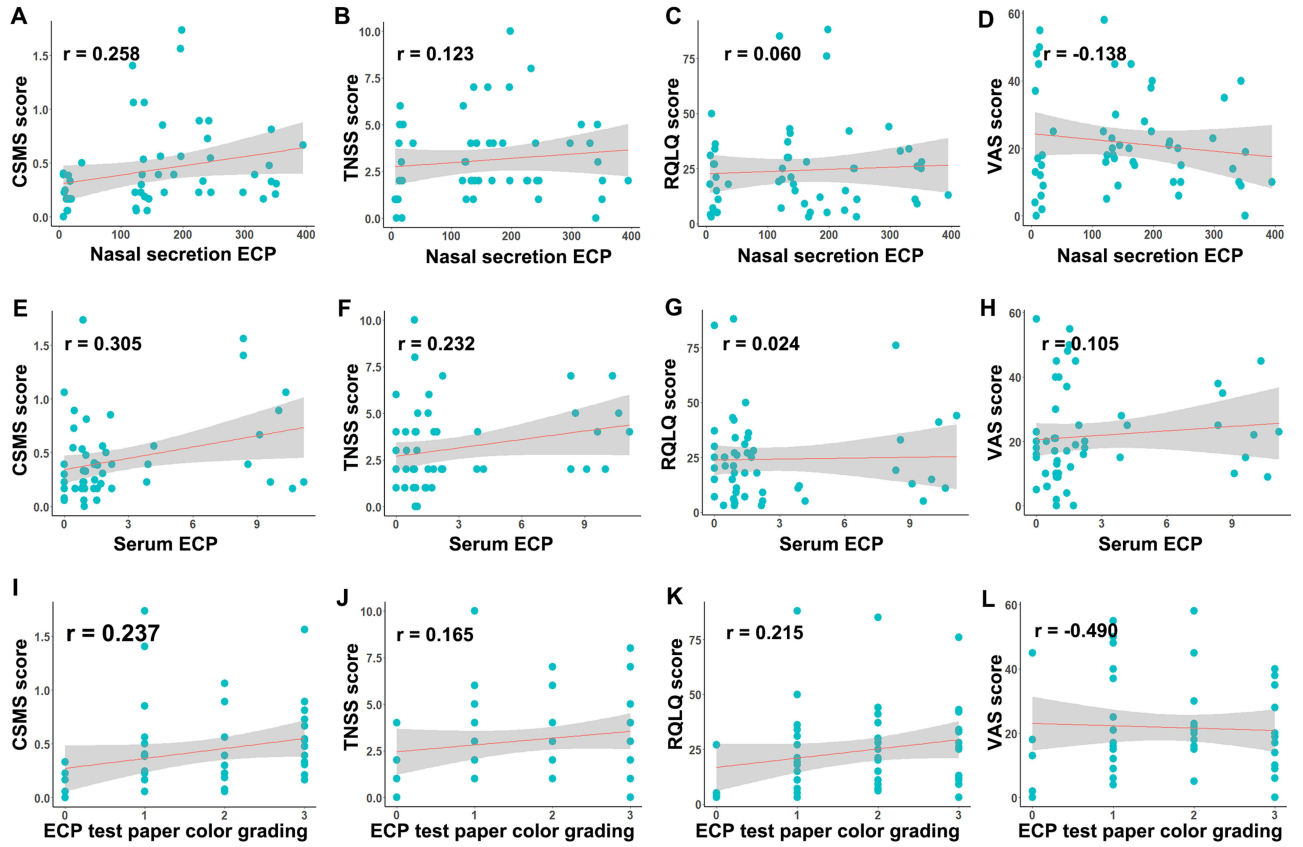


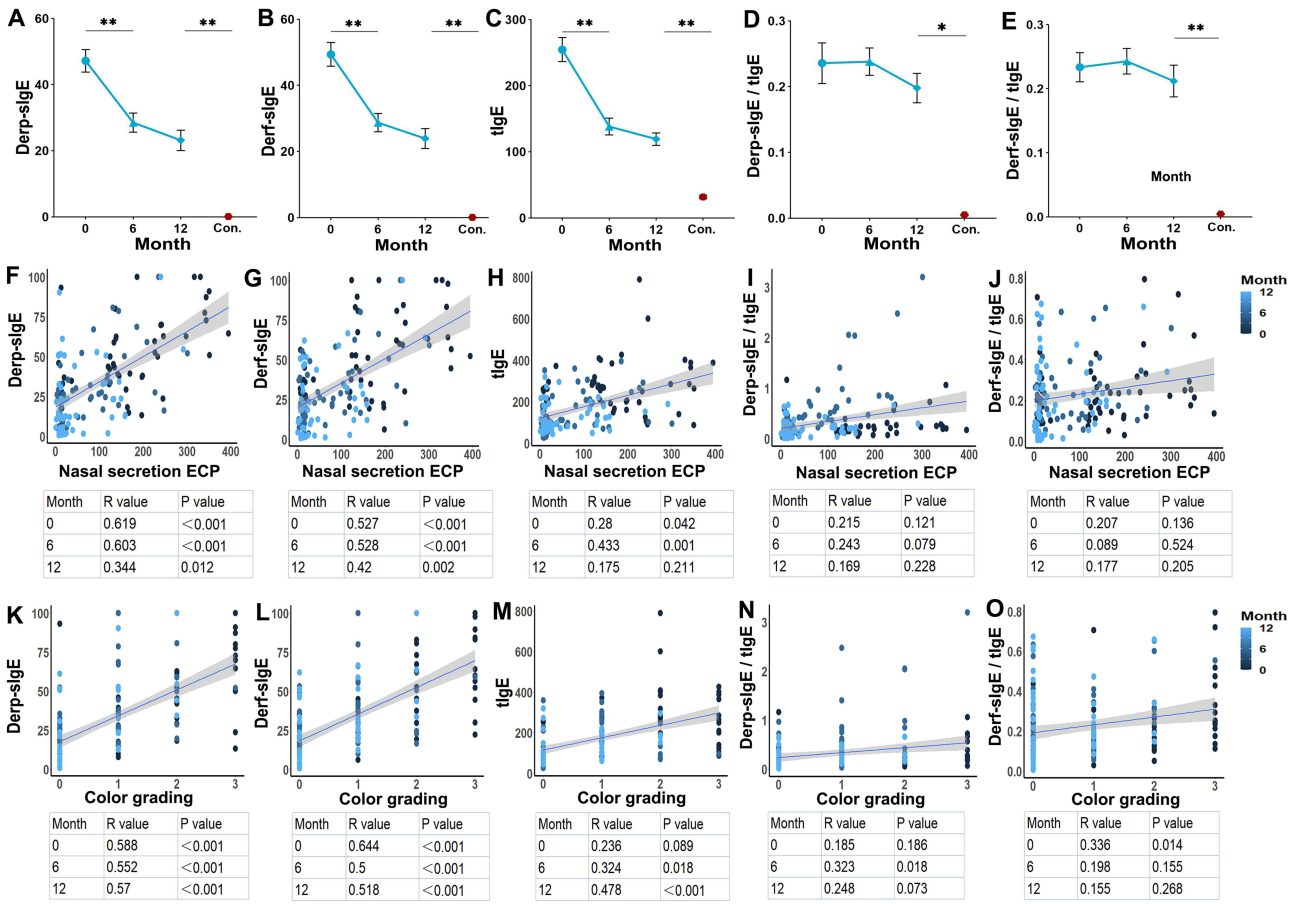
Figure 5 Prognostic function of the test paper for immunotherapy. (**A-D**) Correlation between the concentration of nasal secretion ECP at baseline and clinical symptom score at 12 month. (**E-H**) Correlation between the concentration of serum ECP at baseline and clinical symptom score at 12 month. (**I-L**) Correlation between the color grading of the ECP test paper at baseline and clinical symptom score at 12 month.

Abbreviation: ECP, Eosinophil cationic protein; CSMS, combined symptom and medication; TNSS, Total nasal symptom score; RQLQ, Rhinitis quality of life questionnaire; VAS, Visual analog scale.

most effective treatment for AR.[18] However, the standard therapeutic effectiveness of immunotherapy has not yet been recognized, and a considerable number of indicators are difficult to detect in clinical practice. In this study, 53 patients with AR and corresponding control patients were selected to evaluate the diagnostic effectiveness of the nasal secretion ECP test paper. Here, we explored the clinical effectiveness and prognosis of subcutaneous immunotherapy in patients with AR. We found that the nasal secretion ECP test paper could effectively diagnose AR. Moreover, the results of the test papers were parallel to the symptom score, serum IgE and IgG4 levels, and Th2 inflammatory factors in the process of subcutaneous immunotherapy, indicating that the nasal secretion ECP-MPO test paper results in this study can be used as auxiliary immune markers in the process of subcutaneous immunotherapy for AR.

First, we explored the effectiveness of the nasal secretion ECP test paper for detecting ECP concentrations. In this study, we confirmed that there was a strong positive correlation between the concentration of ECP in nasal secretions and the color grading of the ECP test holes. The eosinophil count of nasal secretion smears positively correlated with the chromogenic grade of the test paper. This shows that the research and development of an ECP real-time test paper is effective, as it can sensitively detect ECP in nasal secretions, and the chromogenic grade of the test paper will increase with an increase in ECP concentration in nasal secretions.

We then verified the diagnostic performance of nasal secretion ECP test papers in patients with AR and corresponding controls. This study found that the sensitivity of the ECP test paper for the diagnosis of AR patients was 90.57%, and the sensitivity was 89.29%. Similarly, using the kappa diagnostic consistency test, this study found that the ECP test paper was highly consistent with the clinical diagnosis. In a study of Korean adult patients with AR, the average serum ECP concentration was 18.8 μg/mL.[19] In this study, the ECP test paper was positive, the cutoff value for ECP concentration in



Month	R value	P value
0	0.619	<0.001
6	0.603	<0.001
12	0.344	0.012

Month	R value	P value
0	0.527	<0.001
6	0.528	<0.001
12	0.42	0.002

Month	R value	P value
0	0.28	0.042
6	0.433	0.001
12	0.175	0.211

Month	R value	P value
0	0.215	0.121
6	0.243	0.079
12	0.169	0.228

Month	R value	P value
0	0.207	0.136
6	0.089	0.524
12	0.177	0.205

Month	R value	P value
0	0.588	<0.001
6	0.552	<0.001
12	0.57	<0.001

Month	R value	P value
0	0.644	<0.001
6	0.5	<0.001
12	0.518	<0.001

Month	R value	P value
0	0.236	0.089
6	0.324	0.018
12	0.478	<0.001

Month	R value	P value
0	0.185	0.186
6	0.323	0.018
12	0.248	0.073

Month	R value	P value
0	0.336	0.014
6	0.198	0.155
12	0.155	0.268

Figure 6 Changes and correlation analysis of serum IgE antibodies. (**A-E**) Clinical changes during immunotherapy (Der p-IgE, Der f-IgE, tIgE, Der p-IgE/tIgE and Der f-IgE/tIgE). (**F-J**) Correlation between the concentration of nasal secretion ECP and Der p-IgE, Der f-IgE, tIgE, Der p-IgE/tIgE and Der f-IgE/tIgE. (**K-O**) Correlation between the color grading of the ECP test paper and Der p-IgE, Der f-IgE, tIgE, Der p-IgE/tIgE and Der f-IgE/tIgE. ECP, Eosinophil cationic protein; tIgE, Total IgE; Der p, Dermatophagoides pteronyssinus; Der f, Dermatophagoides farinae; Con, Control group. *, P<0.05; **, P<0.01.

nasal secretions was 19.31 μg/mL. In addition, we found that when the ECP test paper was positive, the cutoff value for the eosinophil count was 1.55 per high magnification visual field. This indicates that the test paper can detect patients whose ECP concentration in nasal secretions is higher than 19.31ug / mL and / or more than 1.55 eosinophils per high magnification visual field.

Most importantly, this study analyzed the role of test papers in immunotherapy. With the progress of subcutaneous immunotherapy, the color grading of ECP test papers and the concentration of ECP in nasal secretions decreased significantly. The serum ECP concentration also showed a downward trend, but this was not statistically significant. Moreover, according to the ROC of the desensitization time to evaluate the diagnostic function of the ECP test paper turning negative, the area under the curve was 0.7422. When the ECP test paper turned negative, the cutoff value for treatment time was 4.5 months. This cutoff value can provide a theoretical basis for testing strips to monitor the immunotherapy process. The gold standard for immunotherapy efficacy is the assessment of clinical symptoms and medication scores during natural allergen exposure, as defined by the European Academy of Allergy & Clinical Immunology working group according to regulatory guidelines. The main recommended outcome indicators are daily CSMS in AR.[6] In a prospective, open-label, single-center study, patients receiving subcutaneous immunotherapy had significantly lower TNSS at 6 months, 1 year, and 2 years after treatment than at baseline.[20] A meta-analysis showed that, according to RQLQ assessment, the quality of life of patients with subcutaneous immunization improved, which may be the most commonly used and recognized quality of life indicator for evaluating the impact of AR symptoms.[21] In a prospective study of *Dermatophagoides farinae* sensitization and monitoring of the efficacy of subcutaneous

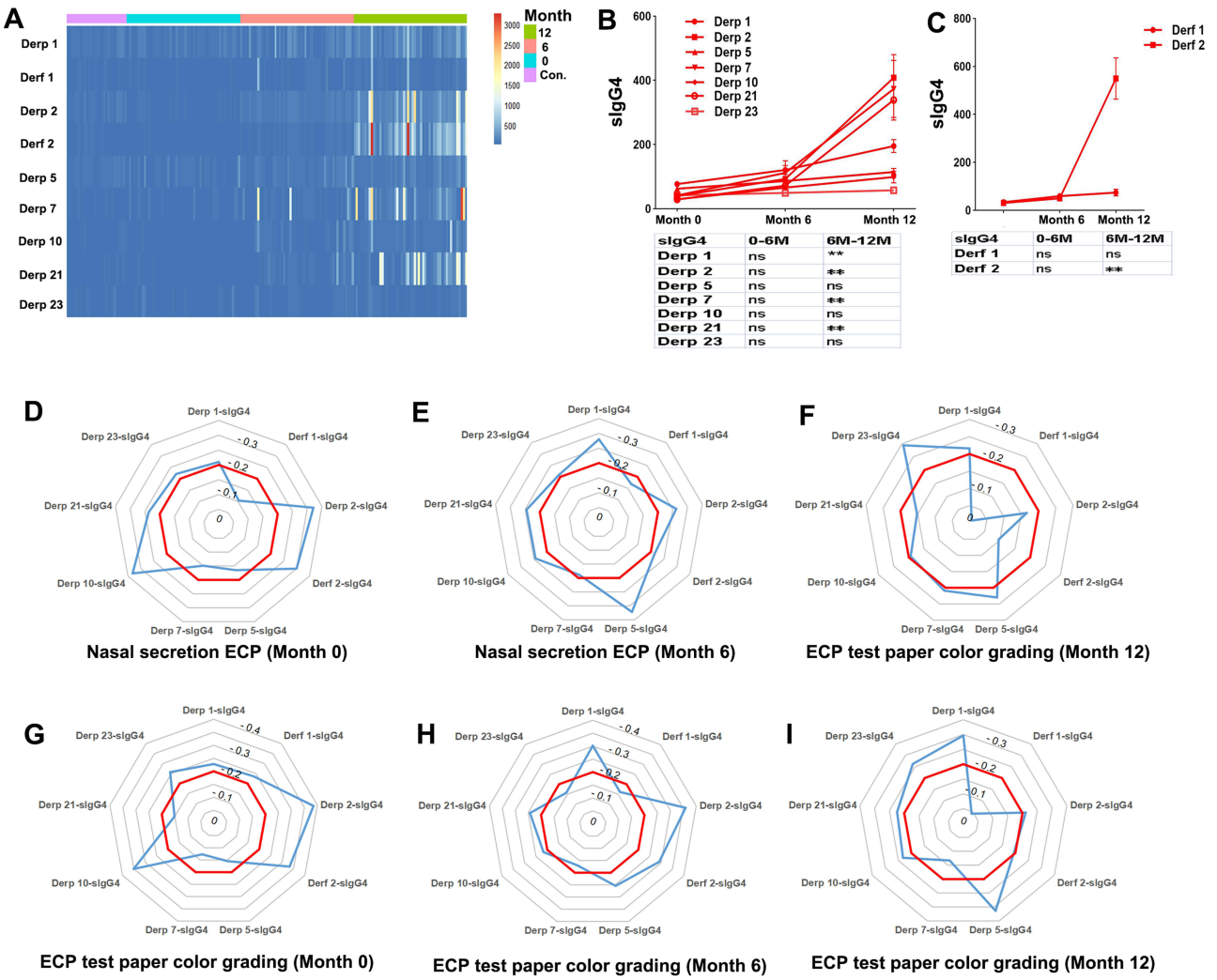


Figure 7 Changes and correlation analysis of serum IgG4 antibodies. (**A**) Heatmap visualization of sIgG4 level to dust mite components of the participants at 0, 6 and 12 months. (**B** and **C**) The changes in sIgG4 to dust mite components during immunotherapy. (**D–I**) Radar image showing the correlation between each dust mite component of sIgG4 and the concentration of nasal secretion ECP, color grading of the ECP test paper at 0, 6 and 12 months. The red circle indicates r = −0.2. ECP, Eosinophil cationic protein; Der p, Dermatophagoides pteronyssinus; Der f, Dermatophagoides farinae; sIgG4, Specific immunoglobulin G4; Con, Control group.

immunotherapy, VAS scores decreased significantly after immunotherapy compared to the control group. Similarly, in this study, it was found that with the advancement of immunotherapy time, CSMS, TNSS, RQLQ, and VAS scores gradually decreased. In this study, it was found that the r correlation coefficients between the scores of CSMS, TNSS, RQLQ, VAS and the color grading of nasal secretions test paper were all more than 0.3, and the positive correlation was good. However, the correlation coefficient between clinical symptom score and serum ECP concentration was less than 0.3, indicating that there was no correlation between them. This shows that the concentration of ECP in nasal secretions, rather than the concentration of ECP in serum, can reflect the severity of AR symptoms, and the color degree of the ECP test paper can also reflect the changes in clinical symptoms in patients with AR during subcutaneous immunotherapy. This further demonstrated their potential as biomarkers of AIT efficacy and patient responsiveness. We also explored the correlation between the color grade of the ECP test hole at the baseline and the clinical symptom score of the patients after 12 months of AIT, but unfortunately no positive results were found, suggesting that the nasal secretion ECP-MPO real-time test paper can not be used to predict the responders and non-responders of AIT.

In the process of subcutaneous immunotherapy, objective laboratory examinations are particularly important in addition to the use of subjective symptoms to evaluate efficacy. We further analyzed the correlation between paper grading and more recognized biomarkers. Elevated serum sIgE levels and symptoms of allergen exposure are the only

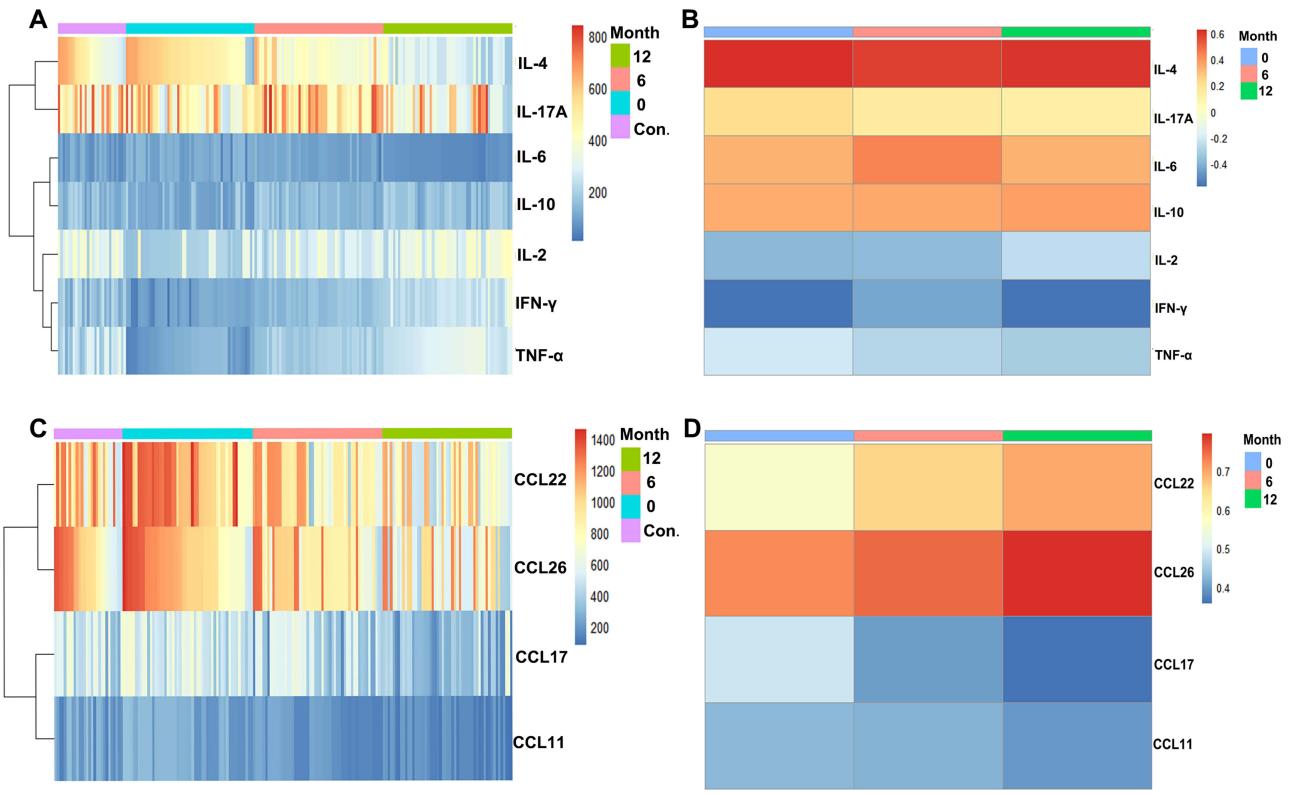


Figure 8 Changes and correlation analysis of serum cytokines and chemokines. (**A**) Clinical changes during immunotherapy (IL-4, IL-17A, IL-6, IL-10, IL-2, IFN-γ and TNF-α). (**B**) Correlation between the color grading of the ECP test paper and IL-4, IL-17A, IL-6, IL-10, IL-2, IFN-γ and TNF-α. (**C**) Clinical changes during immunotherapy (CCL22, CCL26, CCL17 and CCL11). (**D**) Correlation between the color grading of the ECP test paper and CCL22, CCL26, CCL17 and CCL11. Con, Control group.
Abbreviation: IL, Interleukin. CCL, chemokines.

criteria for allergy diagnosis and inclusion criteria for starting immunotherapy.[22] Immunotherapeutic studies have reported that sIgE levels briefly increase during treatment. Long-term immunotherapy studies have shown that sIgE levels decrease over time.[23] In this study, compared with the baseline, the sIgE levels against Der p and Der f decreased significantly after 6 and 12 months of immunotherapy. Unfortunately, no change in the sIgE/tIgE ratio was observed in this study. Moreover, the correlation coefficient between the color grade of nasal secretion ECP test paper and tIgE and sIgE/tige is only about 0.2. This suggests that the correlation between nasal secretion ECP test paper and tIgE is poor, which may be related to the increase of tIgE is not the specific performance of AR. Studies have shown that the total IgE level decreases with the increase of age, and the total IgE level is significantly correlated with occupation type, obesity, smoking status and other factors. The total IgE level is also affected by the ecological imbalance of inferior turbinate mucosal microflora, especially Staphylococcus aureus.[24] The production of serum-specific IgG4 competitively blocks IgE binding to the antigen.[25] Component analysis diagnosis makes accurate drug use and individualized management possible, and further detection of dust mite component sIgG4 in this study. Consistent with other studies, we found that AR patients during immunotherapy were sensitive to the main components of dust mites, Der p1, Der p2 and Der f2. Compared with the sixth month, the serum concentrations of Der p1-sIgG4, Der p2-sIgG4 and Der f2-sIgG4 in the 12th month showed an upward trend. At the same time, there are also increases in Der p7 and Der p21. These two components have been reported as secondary HDM allergens in many studies.[23] According to the special report of the European Institute of Allergy and Clinical Immunology, sIgG4 is recommended as an indicator of patients' compliance with AIT treatment.[6] The concentration of ECP in nasal secretions and the color grading of the test paper were weakly negatively correlated with IgG4 levels against dust mites, especially with Derp 1-IgG4 and Derp 2-IgG4, and the correlation coefficient was about-0.3. These results suggest that immunotherapy not only affects serum IgE and IgG4 antibodies but also affects the concentration of ECP in local nasal secretions. Importantly, this further confirms that the efficacy of

immunotherapy can be judged by using nasal secretion ECP test paper, but the ability to evaluate patients' treatment compliance may be weak.

One hypothetical mechanism for the long-term clinical tolerance after immunotherapy is the transition from a Th2 response to a Th1 response, in which Th2 cytokines are downregulated and Th1 cytokines are up-regulated.[26] The main cytokines IL-10 inhibit the production of IgE through the interaction between Treg cells and B cells.[10] In addition, innate lymphocytes that produce IL-10 can reduce type 2 inflammation.[27] Some studies have shown that immunotherapy may induce Treg function by upregulating Th2 cells.[28] In fact, there was also a decrease in serum eosinophil chemokine levels.[29] Changes in allergen-specific T and B cell responses, decrease in IgE, increase in IgG4 production, and decrease in mast cell and basophil activation thresholds are the main results of successful AIT (Figure 9).[10] In this study, we found that during immunotherapy, Th2 cytokines IL-4 and IL-6 decreased, and cytokine IL-10 showed an upward trend and was positively correlated with the grade of the ECP test paper. The levels of Th1 cytokines IL-2, IFN-γ, and TNF-α were negatively correlated with the grading of ECP test paper. Chemokine CCL26, CCL11, CCL17, and CCL22 levels decreased significantly and were positively correlated with the grade of the ECP test paper. The positive correlation between Th2 cytokine levels and test paper grading indicates that immunotherapy may reduce eosinophil aggregation and ECP release by inhibiting Th2 cell function.

In summary, the nasal secretion ECP-MPO test paper can effectively diagnose AR. In the process of subcutaneous immunotherapy in patients with AR, the grading of nasal ECP test papers has a good correlation with clinical response and has a certain correlation with immunotherapy biomarkers such as serum dust mite IgE and dust mite component IgG4, which can assist in the evaluation of immunotherapy. Compared to the detection of serum sIgE, sIgG4, and Th2-related factors, the nasal secretion test paper is simpler and more rapid, and the results can be read in ten minutes, which saves time in clinical diagnosis and treatment. In the future, multicenter, large-sample clinical trials will be conducted to further evaluate the stability and effectiveness of the test papers for evaluating the efficacy of immunotherapy. More

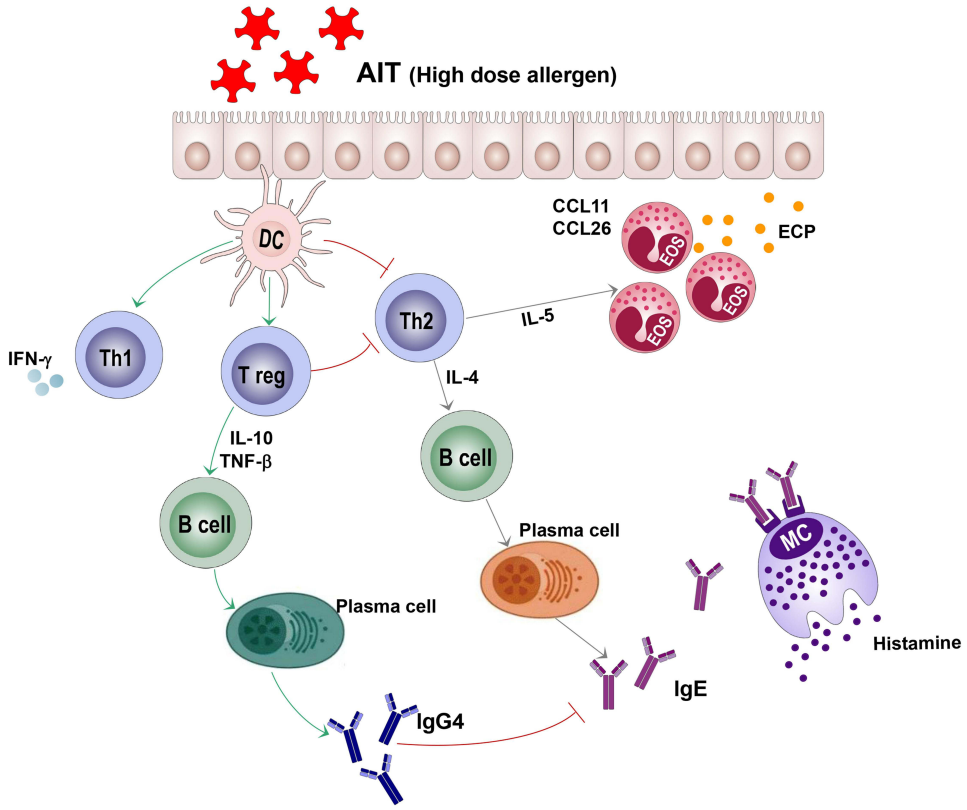


Figure 9 The mechanism of immunotherapy and its relationship with eosinophil cationic protein.
Abbreviation: AIT, Allergen Immunotherapy; DC, Dendritic cell; Th, T helper cell; Treg, Regulatory T cell; IgE, Immunoglobulin E; IgG4, Immunoglobulin G4; IL, Interleukin; ECP, Eosinophil cationic protein; CCL, Chemokines; MC, Mast cell.

accurate test papers will also be developed, ranging from qualitative to semi-quantitative to fully quantitative, which is expected to become a powerful method for evaluation of the efficacy of subcutaneous immunotherapy for AR.

Data Sharing Statement

Any scientist can get access to deidentified database via an e-mail to corresponding author (Ze-Zhang Tao; E-mail: taozezhang696@163.com) upon a reasonable request.

Ethics Statement

The research program was approved by the Ethics Committee of Renmin Hospital of Wuhan University (WDRY2021-K052) and registered in Chinese Clinical Trial Registry (NO. ChiCTR2200056960).

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

1. Hellings P W, Klimek L, Cingi C. et al. Non-allergic rhinitis: position paper of the European Academy of Allergy and Clinical Immunology. *Allergy*. 2017;72(11):1657–1665. doi:10.1111/all.13200
2. Dykewicz MS, Wallace DV, Amrol DJ. Rhinitis 2020: a practice parameter update. *J Allergy Clin Immunol*. 2020;146(4):721–767. doi:10.1016/j.jaci.2020.07.007
3. Bystrom J, Amin K, Bishop-Bailey D. Analysing the eosinophil cationic protein--a clue to the function of the eosinophil granulocyte. *Respir Res*. 2011;12(1):10. doi:10.1186/1465-9921-12-10
4. Tomassini M, Magrini L, De Petrillo G, et al. Serum levels of eosinophil cationic protein in allergic diseases and natural allergen exposure. *J Allergy Clin Immunol*. 1996;97(6):1350–1355. doi:10.1016/S0091-6749(96)70204-X
5. Drazdauskaite G, Layhadi JA, Shamji MH. Mechanisms of Allergen Immunotherapy in Allergic Rhinitis. *Curr Allergy Asthma Rep*. 2020;21(1):2. doi:10.1007/s11882-020-00977-7
6. Shamji M H, Akdis M, Akdis M, et al. Biomarkers for monitoring clinical efficacy of allergen immunotherapy for allergic rhinoconjunctivitis and allergic asthma: an EAACI Position Paper. *Allergy*. 2017;72(8):1156–1173. doi:10.1111/all.13138
7. Nouri-Aria KT, Wachholz PA, Francis JN. Grass pollen immunotherapy induces mucosal and peripheral IL-10 responses and blocking IgG activity. *J Immunol*. 2004;172(5):3252–3259. doi:10.4049/jimmunol.172.5.3252
8. Di Lorenzo G, Mansueto P, Pacor ML, et al. Evaluation of serum s-IgE/total IgE ratio in predicting clinical response to allergen-specific immunotherapy. *J Allergy Clin Immunol*. 2009;123(5):1103–1110,1110–1111. doi:10.1016/j.jaci.2009.02.012
9. Kappen J, Abubakar-Waziri H, Abubakar-Waziri H, et al. Nasal allergen-neutralizing IgG(4) antibodies block IgE-mediated responses: novel biomarker of subcutaneous grass pollen immunotherapy. *J Allergy Clin Immunol*. 2019;143(3):1067–1076. doi:10.1016/j.jaci.2018.09.039
10. Ozdemir C, Cevhertas L, Cevhertas L, et al. Mechanisms of allergen-specific immunotherapy and allergen tolerance. *Allergol Int*. 2020;69(4):549–560. doi:10.1016/j.alit.2020.08.002
11. Tsai Y-G, Yang KD, Wen Y-S. Allergen-specific immunotherapy enhances CD8 + CD25 + CD137 + regulatory T cells and decreases nasal nitric oxide. *Pediatr Allergy Immunol*. 2019;30(5):531–539. doi:10.1111/pai.13061
12. Aroca R, Chamorro C, Vega A, et al. Immunotherapy reduces allergen-mediated CD66b expression and myeloperoxidase levels on human neutrophils from allergic patients. *PLoS One*. 2014;9(4):e94558. doi:10.1371/journal.pone.0094558

13. Montuschi P, et al. Exhaled breath condensate, nasal eosinophil cationic protein level and nasal cytology during immunotherapy for cypress allergy. *J Biol Regul Homeost Agents*. 2013;27(4):1083–1089.
14. Xi Y, D LH, Li H-D, et al. Diagnostic Value of a Novel Eosinophil Cationic Protein-Myeloperoxidase Test Paper Before and After Treatment for Allergic Rhinitis. *J Asthma Allergy*. 2022;15:1005–1019. doi:10.2147/JAA.S375069
15. Obuchowski NA, Zhou XH. Prospective studies of diagnostic test accuracy when disease prevalence is low. *Biostatistics*. 2002;3(4):477–492. doi:10.1093/biostatistics/3.4.477
16. Li J, Fine J. On sample size for sensitivity and specificity in prospective diagnostic accuracy studies. *Stat Med*. 2004;23(16):2537–2550. doi:10.1002/sim.1836
17. Shah SN, Grunwell JR, Mohammad AF. Performance of Eosinophil Cationic Protein as a Biomarker in Asthmatic Children. *J Allergy Clin Immunol Pract*. 2021;9(7):2761–2769. doi:10.1016/j.jaip.2021.02.053
18. Zhang Y, Lan F, Zhang L. Advances and highlights in allergic rhinitis. *Allergy*. 2021;76(11):3383–3389. doi:10.1111/all.15044
19. Min HJ, Hong YH, Yang HS, Kim KS. The correlation of serum eosinophil cationic protein level with eosinophil count, and total IgE level in Korean adult allergic rhinitis patients. *Asian Pac J Allergy Immunol*. 2016;34(1):33–37. doi:10.12932/AP0746
20. Liu W, Zeng Q, He C, et al. Compliance, efficacy, and safety of subcutaneous and sublingual immunotherapy in children with allergic rhinitis. *Pediatr Allergy Immunol*. 2021;32(1):86–91. doi:10.1111/pai.13332
21. Zajac AE, Adams AS, Turner JH. A systematic review and meta-analysis of probiotics for the treatment of allergic rhinitis. *Int Forum Allergy Rhinol*. 2015;5(6):524–532. doi:10.1002/alr.21492
22. W BA, A CM, Casale T, et al. Update on allergy immunotherapy: American Academy of Allergy, Asthma & Immunology/European Academy of Allergy and Clinical Immunology/PRACTALL consensus report. *J Allergy Clin Immunol*. 2013;131(5):1288–1296. doi:10.1016/j.jaci.2013.01.049
23. Yang L, Yang Y, Xu Q, et al. Specific IgE and IgG4 Profiles of House Dust Mite Components in Allergen-Specific Immunotherapy. *Front Immunol*. 2021;12:786738. doi:10.3389/fimmu.2021.786738
24. Hyun DW, Min HJ, Kim MS, et al. Dysbiosis of Inferior Turbinate Microbiota Is Associated with High Total IgE Levels in Patients with Allergic Rhinitis. *Infect Immun*. 2018;86(4):e00934–17. doi:10.1128/IAI.00934-17
25. Okamoto S, Taniuchi S, Sudo K, et al. Predictive value of IgE/IgG4 antibody ratio in children with egg allergy. *Allergy Asthma Clin Immunol*. 2012;8(1):9. doi:10.1186/1710-1492-8-9
26. Akdis CA, Akdis M. Mechanisms of allergen-specific immunotherapy and immune tolerance to allergens. *World Allergy Organ J*. 2015;8(1):17. doi:10.1186/s40413-015-0063-2
27. Golebski K, Layhadi J A, Sahiner U, et al. Induction of IL-10-producing type 2 innate lymphoid cells by allergen immunotherapy is associated with clinical response. *Immunity*. 2021;54(2):291–307. doi:10.1016/j.immuni.2020.12.013
28. Xian M, Feng M, Dong Y, et al. Changes in $CD4^{+}CD25^{+}FoxP3^{+}$ Regulatory T Cells and Serum Cytokines in Sublingual and Subcutaneous Immunotherapy in Allergic Rhinitis with or without Asthma. *Int Arch Allergy Immunol*. 2020;181(1):71–80. doi:10.1159/000503143
29. Jahnz-Rozyk K, Targowski T, Glodzinska-Wyszogrodzka E, et al. Cc-chemokine eotaxin as a marker of efficacy of specific immunotherapy in patients with intermittent IgE-mediated allergic rhinoconjunctivitis. *Allergy*. 2003;58(7):595–601. doi:10.1034/j.1398-9995.2003.00083.x