

Effect of anti-immunoglobulin E on nasal inflammation in patients with seasonal allergic rhinoconjunctivitis

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Summary

Background Binding of allergens to IgE on mast cells and basophils causes release of inflammatory mediators in nasal secretions.

Objective The combined effect of specific immunotherapy (SIT) and omalizumab, a humanized monoclonal anti-IgE antibody, on release of eosinophilic cationic protein (ECP), tryptase, IL-6, and IL-8 in nasal secretion was evaluated.

Methods Two hundred and twenty five children (aged 6–17 years) with a history of seasonal allergic rhinoconjunctivitis induced by birch and grass pollen were randomized into four groups: either birch- or grass-pollen SIT in combination with either anti-IgE or placebo. Complete sets of nasal secretion samples before treatment Visit 1 (V1), during birch- (V2) and grass (V3)-pollen season and after the pollen season (V4) were collected from 53 patients.

Results A significant reduction in tryptase only was seen in the anti-IgE-treated group at V2 ($P < 0.05$) and V4 ($P < 0.05$) compared with the placebo group. During the pollen season, patients with placebo showed an increase of ECP compared with baseline (V2: $+30.3 \mu\text{g/L}$; V3: $+134.2 \mu\text{g/L}$, $P < 0.005$; V4: $+79.0 \mu\text{g/L}$, $P < 0.05$), and stable levels of tryptase, IL-6 and IL-8. Treatment with anti-IgE resulted in stable ECP values and a significant decrease of tryptase compared with V1 (baseline): V2: $-80.0 \mu\text{g/L}$ ($P < 0.05$); V3: $-56.3 \mu\text{g/L}$, which persisted after the pollen season with V4: $-71.6 \mu\text{g/L}$ ($P < 0.05$). After the pollen season, a decrease of IL-6 was observed in both groups (V4 placebo group: -37.5 ng/L ; V4 anti-IgE group: -42.9 ng/L , $P < 0.01$).

Conclusion The combination of SIT and anti-IgE is associated with prevention of nasal ECP increase and decreased tryptase levels in nasal secretions.

Keywords anti-IgE, ECP, IL-6, IL-8, nasal inflammation, seasonal allergic rhinitis, specific immunotherapy, tryptase

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Introduction

Allergic rhinitis (AR) represents a global health problem that affects at least 10–25% of the population and its prevalence is increasing [1–4]. It alters the social life of patients, affects school performance and work productivity and incurs substantial costs. AR is clinically defined as a symptomatic disorder of the nose, induced after allergen exposure, by IgE-mediated inflammation of the nasal membranes. Environmental allergens trigger a cross-linking of specific IgE receptors on mast cells and basophils resulting in a cascade of cell degranulation with the release and synthesis of inflammatory mediators in susceptible individuals [4]. Nasal biopsy studies show accumulation of eosinophils within the lamina propria and epithelium, and an increase in tissue and cell surface basophils in seasonal allergic rhinitis (SAR) [5].

Nasal secretion samples are used to recover nasal inflammatory mediators such as eosinophilic cationic protein (ECP), tryptase, IL-6 or IL-8, and are commonly used for studying inflammatory reactions after allergen challenge in patients with AR. Roca-Ferrer et al. [6] investigated the effect of pro-inflammatory mediators like ECP and IL-8 on airway hypersecretion and demonstrated the direct link between inflammatory mediators and symptom-causing pathophysiologic findings. Furthermore, the coexistence of rhinosinusitis and asthma has been noted in the medical literature [7]. More than 50% of patients with sinus disease also have asthma, and up to 80% of patients with asthma have rhinitis [8]. AR often precedes asthma symptomatology and has been shown to be a risk factor for the development of asthma [9]. Nasal sinus disease may contribute to poor control of asthma [10] and severe sinus disease has been identified as an independent factor associated with frequent severe asthma exacerbations in a recent study [11].

In addition to allergen avoidance and symptomatic therapy, the specific immunotherapy (SIT) can be an effective

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treatment for allergic rhinoconjunctivitis [12, 13], even in the long term [14]. However, the clinical effect may be incomplete in the first year of therapy, and there is a risk of systemic anaphylactic reactions [15, 16]. Complete allergic disease control is infrequently achieved with immunotherapy and conventional pharmacotherapy, particularly for polysensitized patients, who receive SIT with multiple allergens. Recently an anti-IgE antibody has been developed that reduces IgE-mediated symptoms [17] regardless of the allergen specificity of the IgE involved. It lowers levels of serum-free IgE by binding to the specific Fc portion of circulating IgE [18]. To date, a number of clinical studies have demonstrated the safety and efficacy of anti-IgE treatment for AR [17, 19] and asthma [20–22] in both adults and children. As the rhinitis symptoms are caused by nasal mediator release [6], we hypothesized that anti-IgE treatment affects nasal inflammatory mediator levels. In the current study, we measured levels of nasal ECP, tryptase, IL-6 and IL-8 in children with SAR who were treated with either SIT plus anti-IgE or SIT plus placebo.

Methods

Population

In a multi-centre, randomized, double-blind, placebo-controlled, parallel-group study on the effect of anti-IgE in patients with SAR, 225 children and adolescents were recruited from the outpatient departments of 17 centres [19]. Patients were included if they were aged between 6 and 18 years, had a history of birch- and grass-pollen-induced SAR during at least the previous 2 years, and had IgE specific for birch and grass pollen (≥ 0.7 U/L, CAP class ≥ 2 , Pharmacia, Uppsala, Sweden). Subjects with perennial AR, or with total serum IgE levels outside the range 30–1300 IU/mL and body weight > 100 kg (for reasons of dosing) were excluded. In our sub-analysis we investigated the effect of anti-IgE on nasal inflammation in a cohort of 120 patients. Nasal secretion samples from Visits 2 (V2) and 3 (V3) with sampling dates outside the defined pollen seasons, and V4 samples within the pollen season were excluded from the analysis. Out of the original study population we obtained complete sets of nasal secretion samples for V1–4 from 53 patients.

Treatment

Patients were randomized to one of the following treatment groups: grass SIT plus anti-IgE, grass SIT plus placebo, birch SIT plus anti-IgE and birch SIT plus placebo. For details of the SIT see Appendix 1. For both the birch and grass SIT, the build-up phase was started at least 14 weeks prior to the start of the birch-pollen season, with 12 weekly injections into the upper arm, followed by five 4-weekly maintenance injections. The mean cumulative dose of birch SIT allergen extract administered prior to the birch season was 394 300 SQ units, and for grass SIT 478 000 SQ units prior to the grass season. After the build-up phase of SIT (12 weeks, i.e. 2 weeks before expected start of the birch-pollen season), anti-IgE or placebo treatment was started in parallel with SIT. The anti-IgE antibody omalizumab (formerly rhumAb-E25, a recombi-

nant, humanized murine monoclonal IgG1-anti-IgE) or placebo was administered subcutaneously contralateral to SIT every 4 weeks for 24 weeks (anti-IgE dose dependent on body weight and pre-seasonal total IgE level, providing a dose of at least 0.016 mg/kg/IU/mL of total IgE every 4 weeks; for patients with high IgE levels and/or high body weight doses were given at 2-weekly intervals). Clinical status, venous blood sampling, nasal secretion sampling and spirometry were performed before treatment (baseline V1, November and December 1999), during the expected birch- and grass-pollen season in 2000 (V2 and V3) and after the end of treatment (V4). The start and end of the pollen season (a total average of 84 days in this study) were defined by the threshold of > 10 birch grains/m³/24 h and > 5 grass grains/m³/24 h for 3 consecutive days. An assigned study nurse, who did not interact with the treating doctor, administered anti-IgE and placebo injections. Treatment was therefore blinded to both patients and the treating doctor. The timetable of the whole study is shown in Fig. 1. The efficacy results of the main study were published earlier [19]. Signed informed consent was obtained from all subjects and their guardians, and the ethics committees approved the study.

Nasal inflammation mediators

Nasal secretion samples were obtained by inserting cotton gauze strips beneath the lower turbinate for 15 min. After removal, the gauze was removed by centrifugation in special tubes (Salivette, Fa. Sarstedt, Nümbrecht, Germany). The resulting nasal fluid was stored at -80°C until testing. Samples (50 μL) of diluted nasal fluid were analysed fluorometrically for tryptase (Pharmacia), ECP, IL-6 and IL-8 using a two-sided chemiluminescent test (Immulate DPC, Bad Nauheim, Germany).

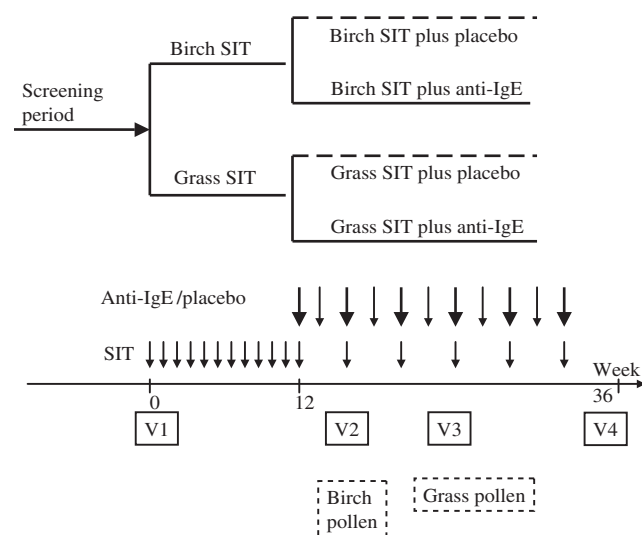


Fig. 1. Study timetable. Clinical status, venous blood sampling, nasal secretion sampling and spirometry were performed at Visits 1–4 (V1–V4). V1: start of specific immunotherapy (SIT) (November/December 1999); V2: during birch-pollen season; V3: during grass-pollen season; V4: after the end of the treatment.

Clinical data

All patients recorded daily symptoms and rescue medication usage in a diary. The daily symptom severity score was the mean severity (using a four-point scale; 0 = none to 3 = severe) of each of the following seven symptoms: itchy, runny, or stuffy nose; sneezing; itchy, watery or red eyes. The daily rescue medication (topical levocabastine, inhaled salbutamol, oral cetirizine or oral prednisolone) score was defined as 0 = no rhinitis medication, 1 = topical nasal, ocular or lung treatment apart from corticosteroids, 2 = oral antihistamines, 3 = oral or topical corticosteroids for nose or lung. The primary parameter of the main study was the symptom load for the entire pollen season. The daily symptom load was the sum of the daily symptom severity score and rescue medication score.

Tolerability and overall safety

As previously published, the tolerability and overall safety were similar in the anti-IgE and placebo groups [19].

Statistical analysis

Only data of patients with complete data sets for V1–4 were analysed. Therefore the number of patients differs from the whole study population. Data were analysed in two ways using SPSS: (1) The differences between the anti-IgE- and placebo-treated groups at every visit were calculated using the *t*-test (Table 3). V1 was baseline and set to zero. Baseline values for ECP, tryptase, IL-6 and IL-8 are shown separately (Table 2). (2) The seasonal course was analysed using the *t*-test for changes from baseline within each group. $P < 0.05$ was regarded as significant.

Results

Clinical data

Table 1 shows age, gender, symptom severity score, rescue medication score, and symptom load for the birch- and grass-

Table 1. Study population characteristics and symptom severity score, rescue medication score and symptom load

	Anti-IgE-treated group	Placebo-treated group
Number of patients	30	23
Age, median (range)	12 (7–17)	11 (7–17)
Male : female	16 : 14	15 : 8
Symptom severity score		
Birch-pollen season	0.10*	0.46
Grass-pollen season	0.16**	0.50
Rescue medication score		
Birch-pollen season	0.06**	0.29
Grass-pollen season	0.0***	0.42
Symptom load		
Birch-pollen season	0.26**	0.75
Grass-pollen season	0.29***	0.92

Score data are shown as median with * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$ between both groups.

pollen season for the two groups. Anti-IgE treatment reduced the symptom severity score (anti-IgE: 0.10 vs. placebo: 0.46, $P < 0.05$ for birch-pollen season, and 0.16 vs. 0.50, $P < 0.01$ for grass-pollen season), rescue medication score (anti-IgE: 0.06 vs. placebo: 0.29, $P < 0.01$ for birch-pollen season; and 0.00 vs. 0.42, $P < 0.0001$ for grass-pollen season), and symptom load (anti-IgE: 0.26 vs. placebo: 0.75, $P < 0.01$ for birch-pollen season; and 0.29 vs. 0.92, $P < 0.0001$ for grass-pollen season) compared with placebo. These data are in accordance with the recently published main study [19].

Nasal ECP

There were no statistically significant differences in ECP between groups at baseline or at any other point during the study (mean at birch-pollen season V2: placebo group 30.3 µg/L, anti-IgE group 10.0 µg/L; at grass-pollen season V3: placebo group 134.2 ng/L, anti-IgE 49.0 µg/L and after the pollen season V4: placebo group 79.0 µg/L, anti-IgE group –13.2 µg/L, Table 3). However, analysis of the seasonal course demonstrates an increase in ECP levels during grass-pollen season (V3) compared with baseline in both groups, but the increase in the placebo group had greater relevance (placebo V3 vs. V1: $P < 0.005$; V4 vs. V1: $P < 0.05$), see Fig. 2a.

Nasal tryptase

Similar levels were observed at V1 in both groups (baseline mean, placebo group 93.5 µg/L, anti-IgE group 133.9 µg/L), see Table 2. Tryptase levels in patients treated with anti-IgE were significantly lower during birch season compared with placebo (mean placebo group 17.6 µg/L, anti-IgE group –80.0 µg/L, $P < 0.05$) and after pollen season (placebo group 40.1 µg/L, anti-IgE group –71.6 µg/L, $P < 0.05$) compared with the placebo-treated group. This was not shown in the grass-pollen season (placebo group 15.5 µg/L, anti-IgE group –56.3 µg/L), see Table 3.

Patients treated with anti-IgE showed decreasing nasal tryptase levels throughout the pollen season (birch-pollen season V2 vs. V1: $P < 0.05$; and after the pollen season V4 vs. V1: $P < 0.05$). Patients treated with placebo showed stable tryptase levels through the pollen season, see Fig. 2b.

Nasal IL-6

Similar IL-6 levels were seen in both groups before treatment (baseline mean; V1: placebo group 101.2 ng/L, anti-IgE group 92.9 ng/L, Table 2). There were no statistically significant differences between the placebo and anti-IgE groups during the study (mean V2: placebo group –5.9 ng/L, anti-IgE group –14.3 ng/L; at grass-pollen season V3: placebo group –10.5 ng/L, anti-IgE group 32.5 ng/L and after the pollen season V4: placebo group –37.5 ng/L, anti-IgE group –42.9 ng/L, Table 3).

Throughout the birch-pollen season and at the end of the study we found a slight reduction in IL-6 levels in both groups, that became significant at the final visit in comparison with baseline ($P < 0.01$ in both groups), see Fig. 2c.

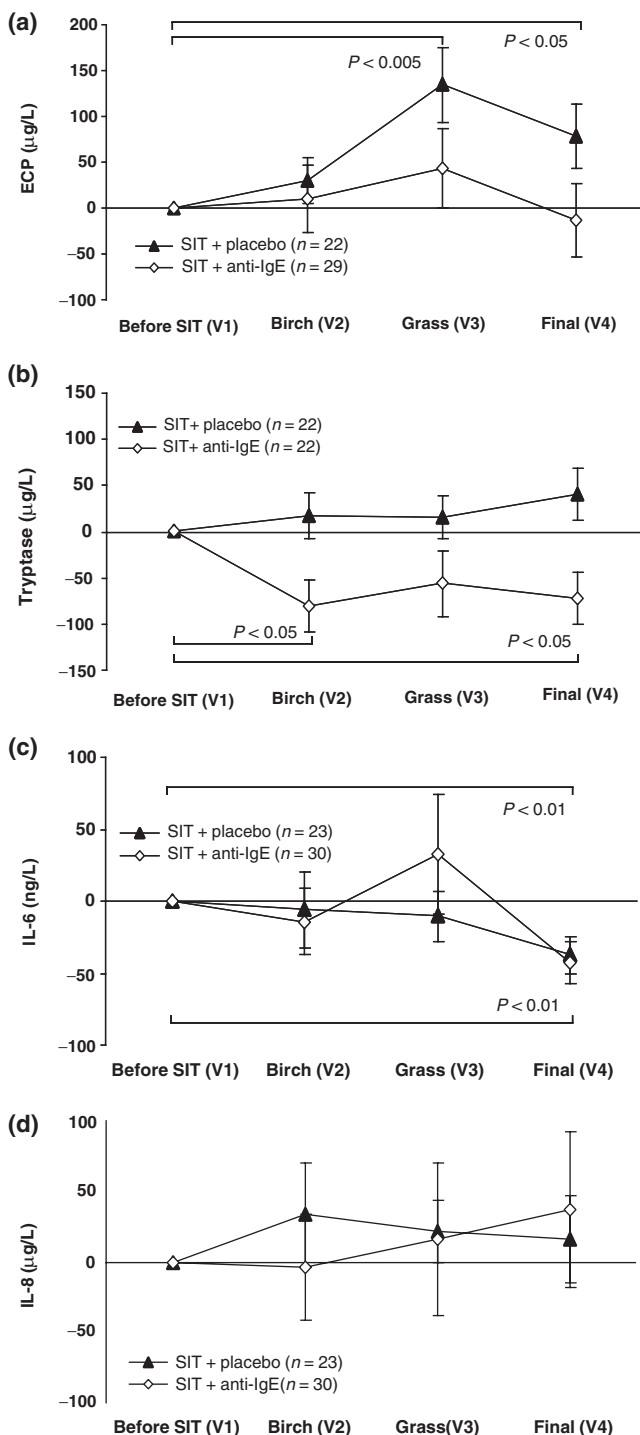


Fig. 2. Changes from baseline for nasal eosinophilic cationic protein (ECP) (a), tryptase (b), IL-6 (c) and IL-8 (d) levels within each group. Only patients with complete data sets are included. Results are shown as mean. V1, Visit 1; V2, Visit 2; V3, Visit 3; V4, Visit 4.

Nasal IL-8

There were no statistically significant differences between placebo and the anti-IgE group at V1 and throughout the study (mean V2: placebo group 33.3 $\mu\text{g/L}$, anti-IgE group $-3.5 \mu\text{g/L}$; at grass-pollen season V3: placebo group 21.8 $\mu\text{g/L}$, anti-IgE group 15.9 $\mu\text{g/L}$; after pollen season V4: placebo group 16.1 $\mu\text{g/L}$, anti-IgE group 37.1 $\mu\text{g/L}$, Table 3).

Table 2. Baseline nasal levels of eosinophilic cationic protein (ECP), tryptase, IL-6, and IL-8 of patients treated with anti-IgE and placebo

Mediator	Treatment	Visit 1 (before treatment)
ECP ($\mu\text{g/L}$)	Placebo	$n = 22, 68.2 (\pm 62.5)$
	Anti-IgE	$n = 29, 101.7 (\pm 176.8)$
Tryptase ($\mu\text{g/L}$)	Placebo	$n = 22, 93.5 (\pm 107.0)$
	Anti-IgE	$n = 22, 133.9 (\pm 174.6)$
IL-6 (ng/L)	Placebo	$n = 23, 101.2 (\pm 96.1)$
	Anti-IgE	$n = 30, 92.9 (\pm 71.4)$
IL-8 ($\mu\text{g/L}$)	Placebo	$n = 23, 19.0 (\pm 50.8)$
	Anti-IgE	$n = 30, 55.5 (\pm 175.1)$

Data are shown as number of patients (n) and mean ($\pm$ SD).

Patients in both groups showed a tendency to increasing IL-8 levels through and after the pollen season compared with baseline (not significant), see Fig. 2d.

Discussion

When a type I hypersensitivity reaction with cross-linking of specific IgE takes place, the degranulation of mast cells is the first event, followed by the release of mediators, cellular infiltration and inflammation where eosinophils are present. The mast-cell degranulation and the activation of eosinophils can be easily demonstrated by the increase of specific mediators, such as ECP, tryptase and ILs [23]. ECP is located within the matrix of specific granules in eosinophil leucocytes and is considered to be a marker of eosinophil activity [24–26], whereas tryptase, a serine endoprotease, is selectively released from mast cells [27]. Increased levels of ECP, tryptase and IL-6 in nasal secretion samples and lavages have been found in patients with AR during allergen exposure [28–30].

The aim of our subanalysis was to evaluate the effect of anti-IgE on nasal inflammation in patients allergic to birch and grass pollen treated with SIT. We investigated ECP, tryptase, IL-6 and IL-8 levels in the nasal secretions of patients receiving SIT plus placebo or SIT plus anti-IgE, before, during and after pollen season. Two different comparisons were made: (1) the difference between anti-IgE- and placebo-treated groups at every visit, and (2) the seasonal course within each treatment group.

There was a significant increase in nasal ECP for the placebo-treated group compared with baseline during the grass-pollen season, but the increase was not significant at the birch-pollen visit. Only slight increases in ECP levels were observed for the anti-IgE-treated group. However, there was no relevant difference between the placebo- and anti-IgE-treated groups at any visit. These apparently conflicting results show that nasal ECP is a useful marker to monitor individual courses of seasonal allergy in patients, but high levels of individual variability were probably the reason why differences between treatment groups were not detected. Additionally, we found that patients treated with anti-IgE experienced decreasing nasal tryptase levels that persisted after the pollen season, whereas patients treated with placebo had stable levels. Furthermore, compared with placebo, anti-IgE-treated patients had significantly reduced nasal tryptase levels both

Table 3. Nasal levels of eosinophilic cationic protein (ECP), tryptase, IL-6, and IL-8 of patients treated with specific immunotherapy (SIT) plus anti-IgE and SIT plus placebo for visits during the birch- and grass-pollen season and after pollen season

Mediator	Treatment	Visit 2 (birch-pollen season)	Visit 3 (grass-pollen season)	Visit 4 (after treatment)
ECP ($\mu\text{g/L}$)	Placebo, $n = 22$	30.3 (± 116.6)	134.2 (± 191.6)	79.0 (± 164.4)
	Anti-IgE, $n = 29$	10.0 (± 200.5)	49.0 (± 233.9)	-13.2 (± 214.4)
Tryptase ($\mu\text{g/L}$)	Placebo, $n = 22$	17.6 (± 118.0)	15.5 (± 106.6)	40.1 (± 133.3)
	Anti-IgE, $n = 22$	-80.0 (± 133.7)*	-56.3 (± 170.1)	-71.6 (± 133.2)*
IL-6 (ng/L)	Placebo, $n = 23$	-5.9 (± 125.4)	-10.5 (± 85.0)	-37.5 (± 63.2)
	Anti-IgE, $n = 30$	-14.3 (± 125.4)	32.5 (± 229.1)	-42.9 (± 80.3)
IL-8 ($\mu\text{g/L}$)	Placebo, $n = 23$	33.3 (± 179.4)	21.8 (± 106.9)	16.1 (± 146.4)
	Anti-IgE, $n = 30$	-3.5 (± 216.8)	15.9 (± 296.7)	37.1 (± 302.9)

Only patients full data set for all visits were included in the analysis. Data are shown as number of patients (n), mean ($\pm$ SD) for the difference in both treatment groups.

* $P < 0.05$.

during and after the birch-pollen season. Anti-IgE neutralizes free IgE, diminishes the IgE production by B cells [31] and down-regulates the receptor Fc ϵ RI [29, 30, 32], which is found at both eosinophil granulocytes and mast cells. The stable nasal ECP levels for anti-IgE-treated patients (but increasing ECP in placebo-treated patients) and reduced tryptase levels under anti-IgE treatment were, therefore, expected. However, the following exceptions need to be explained. Nasal ECP did not increase significantly in the placebo-treated group at the birch-pollen season but did at grass-pollen season. Although patients with sampling dates outside the defined pollen counts were excluded, we speculate that the antigen burden during the birch-pollen season was generally lower than during the grass-pollen season, which could explain a weak ECP increase in placebo-treated patients. Tryptase levels were stable in the placebo-treated group, but decreased in the anti-IgE-treated group. We did not include a healthy control group, but we would suggest that the baseline tryptase levels were already elevated in our patients (as supported by other studies [23]), and normalized under anti-IgE treatment. Differences in tryptase levels between the treatment groups were not significant during the grass-pollen season but were significant during and after the birch-pollen season.

The lower levels of tryptase in nasal secretions from the anti-IgE group suggest that Fc ϵ RI on mast cells and/or the release of mediators are down-regulated with anti-IgE treatment [40]. This is supported by a recent study of Kopp et al. [33] who studied the same patient population and found that anti-IgE treatment reduced the release of sulphido-leukotriens (SLT) in whole blood compared with placebo.

We found a slight reduction in IL-6 levels through the pollen season in both groups that became significant at the final visit compared with baseline. However, increased IL-6 levels after allergen, methacholine and histamine challenges have been described before [34]. All patients were treated with SIT for birch or grass pollen starting 14 weeks before birch-pollen season, so an immune modulating effect of SIT could have reduced IL-6 levels in both groups, with an effective cumulative SIT dose being reached at the final visit, resulting in the significant reduction of IL-6 levels. This hypothetical effect needs to be considered for all of the investigated mediators, as it has been reported recently that ECP and tryptase are down-modulated by SIT [35]. Existing differences between the placebo and anti-IgE-treated groups could also be covered by this effect.

Conflicting results have been reported for nasal IL-8 levels. Some studies reported a significant increase in IL-8 after nasal allergen provocation [36, 37], whereas others found no significant changes in IL-8 or neutrophils in nasal lavage fluid during the pollen season [28, 38]. Another study found that IL-8 levels did not decrease following budesonide treatment, while IL-5 levels and relative eosinophil counts increased significantly during the pollen season [39]. IL-8 levels have been correlated with neutrophil counts but not with eosinophil counts during the pollen season [36]. The addition of a neutralizing anti-IL-8 antibody has been shown to weakly inhibit the chemotactic activity, which suggests that the role of this chemokine in granulocyte infiltrate was limited and that IL-8 acts in connection with other chemotactic factors in neutrophil recruitment. As anti-IgE down-regulates the receptor density on basophils and mast cells [40] and inhibits tryptase release, we might speculate that the slight increase in IL-8 indicates an immune modulating effect of anti-IgE treatment, i.e. an altered profile of IgE-mediated inflammation or a shift to other cells such as macrophages and neutrophils.

While the local levels of inflammatory mediators are known to vary during allergen exposure and treatment, their clinical impact needs to be confirmed. Several studies of cells infiltrating the nasal mucosa during the pollen season found that the increase in inflammatory cells was correlated with both the severity of symptoms [41–44] and nasal non-specific hyper-reactivity [45, 46]. The airway mucosa responds to inflammatory provocations with bulk exudation of plasma into the airway tissue (vascular exudation) and lumen (mucosal exudation [47]). The process of mucosal exudation of plasma is a prominent feature of airway inflammation and has been demonstrated in rhinitis [48]. In this study, treatment with anti-IgE prevented nasal ECP increase and reduced tryptase levels, and significantly improved symptom scores and rescue medication use compared with placebo. This confirms similar findings recently published by Kühr et al. [19]. The impact of anti-IgE treatment on other parts of the airway mucosa such as the bronchial mucosa should also be mentioned. Both nasal and bronchial mucosa are characterized by a pseudostratified epithelium with columnar, ciliated cells resting on a basement membrane [49]. The high degree of vascularization is a key feature of the nasal mucosa and changes in the vasculature may lead to severe nasal obstruction [50]. Smooth muscle, however, is an important

factor in asthma, being present from the trachea to the bronchioles and facilitating bronchoconstriction. A growing number of studies show that the inflammation of nasal and bronchial mucosa is sustained by a similar inflammatory infiltrate [49]. AR frequently precedes the development of asthma [49, 51] and often causes a worsening of asthma. Several different mechanisms are involved including bronchoconstriction causing mediator release, post-nasal mucous drip or mouth breathing [52]. Frequently, patients suffer from both SAR and asthma. Anti-IgE treatment, however, potentially reduces symptoms of both manifestations of allergic airways disease.

In conclusion, monitoring of the individual effect of anti-IgE treatment by nasal secretion sampling is possible, but more studies are needed. We found that SIT plus anti-IgE prevented nasal ECP increase and reduced tryptase levels. At the same time, symptom severity and rescue medication use decreased significantly, confirming similar recent reports [19]. Anti-IgE is independent of allergen specificity, so it is especially suitable for polysensitized allergic patients. Anti-IgE therapy appears to be a very promising treatment for patients suffering from both seasonal allergic rhinoconjunctivitis and asthma.

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Appendix 1

Specific immunotherapy

The birch-pollen extract (ALK-Scherax/Abello, Berlin, Germany) contained 12.3 µg of major allergen (bet v 1) per 100 000 SQ units. The grass-pollen extract provided an average of 14.4 µg of major allergen per 100 000 SQ units, containing (in micrograms of major allergen per 100 000 SQ units): *Phleum pratense* (20.2), *Avena eliator* (12.0), *Dactylis glomerata* (12.0), *Lolium perenne* (12.5), *Poa pratense* (15.2), *Festuca pratense* (18.6) and *Secale cereale* (10.2).