

Pretreatment IgE sensitization patterns determine the molecular profile of the IgG₄ response during updosing of subcutaneous immunotherapy with timothy grass pollen extract

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Background: Allergen immunotherapy is an effective treatment of allergic rhinoconjunctivitis. Clinical efficacy is associated with improvement of basophil sensitivity and an increase in allergen-specific immunoglobulin concentration.

Objective: We sought to determine whether changes in allergen component-specific serum IgE and IgG₄ levels during the updosing phase of subcutaneous immunotherapy (SCIT) are biomarkers of the immunologic changes that can lead to treatment efficacy.

Methods: Twenty-four subjects with grass pollen-induced allergic rhinoconjunctivitis were randomized 3:1 to receive SCIT (Alutard SQ) or to an open control group. IgE and IgG₄ concentrations were determined for the major allergens Phl p 1 or Phl p 5 by using ImmunoCAP and for 8 grass pollen molecules by using Immuno Solid-phase Allergy Chip (ISAC) before treatment and after updosing.

Results: Levels of specific IgE against the dominant major allergens Phl p 1 and Phl p 5 increased from a mean of 23.0 to 48.8 kU/L ($P = .01$, $n = 18$) during the updosing phase in ImmunoCAP measurements but decreased from a median of 4.6 ISAC specific units (ISU) to 2.14 ISU ($P < .0001$, $n = 102$) when measured by using ISAC against 8 grass allergen components. The updosing phase induced a specific IgG₄ level increase from a median of 0 ISU before treatment to 0.83 ISU after 12 weeks ($P < .0001$, $n = 102$) but only for allergen molecules to which pretreatment-specific IgE antibodies were detected (Spearman $\sigma = 0.72$, $P < .0001$, $n = 102$).

Conclusion: Pretreatment allergen component-specific IgE appears to determine the induction of IgG₄ in the updosing phase. Induced IgG₄ seems to suppress IgE levels on ISAC, resulting in a marked decrease in ISAC-measured specific IgE

levels after updosing of SCIT. Thus this decrease in ISAC IgE levels can be used to monitor the blocking effect of allergen-specific immunotherapy-induced non-IgE antibodies. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: Grass pollen allergy, subcutaneous immunotherapy, component-resolved diagnosis, allergen component-specific IgE, allergen component-specific IgG₄, molecular allergy, allergen immunotherapy biomarker

Hay fever caused by grass pollen allergy^{1,2} is a major health problem in industrialized countries. The socioeconomic effect is mainly evident from the loss of productivity at work^{3,4} and poorer performance in school.⁵

Allergen-specific immunotherapy (AIT) is the only treatment modifying the natural history of allergic diseases.^{6,7} It results in complex cellular and humoral changes.⁸ The induction of an allergen-specific IgG₄ response is an early effect of AIT.⁹⁻¹¹

Traditionally, allergen extracts consisting of a complex and partially characterized mixture of allergen molecules¹² are used for both diagnosis and treatment. The recent development of molecular allergy leads to component-resolved diagnosis (CRD), which characterizes individual sensitizations at a molecular level.¹³ To some extent, CRD can demonstrate risk-associated sensitizations and distinguish between primary sensitizations and cross-reactivity.¹⁴

We explored the performance of biomarkers to monitor treatment efficacy in a trial of subcutaneous immunotherapy (SCIT) compared with an open control group. The primary outcome of this study was a significant decrease in basophil sensitivity with a dominant humoral component.¹⁵

In the current analysis we examined changes in allergen component-specific IgE and IgG₄ levels measured by using ImmunoCAP and Immuno Solid-phase Allergy Chip (ISAC) before and after updosing to explain this observed decrease in basophil sensitivity and to identify biomarkers for the immunologic changes that might predict treatment effects of AIT.

METHODS

Study population

We recruited 24 subjects with seasonal rhinoconjunctivitis caused by grass pollen allergy in December 2009, recently described in detail.¹⁵ All participants were examined at the baseline visit. A disease history was obtained, and the participants completed a standardized allergy questionnaire, listing all clinical relevant allergies. Sensitization was confirmed by a skin prick test with timothy grass extract (Soluprick SQ *Phleum pratense*; ALK-Abelló, Hørsholm, Denmark) resulting in a wheal diameter of greater

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Abbreviations used

AIT: Allergen-specific immunotherapy
 CRD: Component-resolved diagnosis
 FAB: Facilitated Antigen Binding
 FAP: Facilitated antigen presentation
 IQR: Interquartile range
 ISAC: Immuno Solid-phase Allergy Chip
 ISU: ISAC specific units
 SCIT: Subcutaneous immunotherapy

than 3 mm. Participants retrospectively indicated their maximum symptom score during the grass pollen season preceding treatment when entering the study and reported daily symptom and medication scores during the grass pollen season after up dosing of SCIT. We used a symptom score combining 4 nasal and 2 ocular symptoms on a scale from 0 to 3.¹⁶ Participants were randomized 3:1 according to a computer-generated list to receive standard SCIT ($n = 18$) or to an open control group ($n = 6$) only receiving reliever medication as needed. All participants provided informed consent when entering the study. The regional ethics committee approved the study (M2009-0121), which meets the CONSORT 2010 guidelines for reporting randomized trials and is registered at ClinicalTrials.gov (NCT01085526).

SCIT

SCIT was initiated with a modified cluster on day 1 (10, 100, and 1000 SQU), followed by injections of increasing doses of timothy grass extract (Alutard SQ *Phleum pratense*, ALK-Abelló) at weekly intervals until a maintenance dose (100,000 SQU) was reached before the 2010 grass pollen season.

Immunoglobulin measurements

Before AIT initiation and after 12 weeks of up dosing treatment, allergen-specific IgE and IgG₄ levels were measured by using 2 different standard methods: quantitative levels of Phl p 5-specific IgE or, if less than the detection limit, Phl p 1-specific IgE and corresponding IgG₄ antibodies were measured by using ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden). Specific IgE and IgG₄ antibody levels to Phl p 1, Phl p 2, Phl p 4, Phl p 5, Phl p 6, Phl p 7 (polcalcin), Phl p 11, and Phl p 12 (profilin) were measured on the semiquantitative 112-allergen ISAC (Thermo Fisher Scientific). Serum samples were frozen at -80°C and processed at the same time.

Data on basophil sensitivity measured by means of flow cytometry and Facilitated Allergen Binding (FAB), an assay measuring the inhibition of allergen binding to B cells used in this analysis, were reported recently.¹⁵

Statistical analysis

Data are reported as means with 95% CIs or medians with interquartile ranges (IQRs). Normal distribution was verified by QQ-plots and histograms. Normally distributed data were analyzed by using a t test for between-group analysis and a paired t test for intraindividual in-group analysis. Otherwise, we used a Mann-Whitney-Wilcoxon test to perform between-group analysis and the Wilcoxon signed-rank test to analyze intraindividual in-group differences. We used a multiple regression model with maximum symptom scores before treatment and after the ensuing season as dependent variables and the sum of ISAC grass-specific IgE and IgG₄ levels, basophil sensitivity, and FAB as explanatory variables both before treatment and after up dosing. The model was checked by plotting residuals. Correlation coefficients were obtained by using the Spearman rank test. For statistical analysis of the suppressive effect of induced IgG₄ on ISAC IgE measurements, we divided all IgG₄ measurements after up dosing into 4 groups. The first had no detectable IgG₄ (<0.03 ISAC-specific units [ISU], $n = 56$), and the remaining 88 measurements were divided into tertiles: 0.04 to 0.50 ISU ($n = 29$); 0.56 to 3.19 ISU ($n = 30$); and 3.22 to 9.98 ISU ($n = 29$), respectively. We plotted

these groups against the changes in IgE concentration between the 2 measurements. The pretreatment IgE measurements were divided into 4 groups according to ISAC classes (not detectable, <0.30 ISU; low, 0.30-0.99 ISU; moderate to high, 1.00-14.99 ISU; and very high, >15.00 ISU) and plotted against changes in IgG₄ concentrations. This is an exploratory study in which a P value of .05 or less was considered statistically significant without correction for multiple tests. All data were analyzed with Stata 11.1 software for Windows (www.stata.com).

RESULTS**Baseline characteristics**

Median duration of grass pollen-induced hay fever at inclusion was 9.5 years (IQR, 5.5-17.5 years). All study participants were sensitized to at least 1 of the major allergens Phl p 1 and Phl p 5 at baseline (Table 1). Ten (41.7%) patients reported hay fever symptoms exclusively caused by grass pollen exposure. Two of these subjects were monosensitized to grass pollen in the pretreatment ISAC measurements. At baseline, participants had detectable IgE against Phl p 2 (54.2%), Phl p 4 (54.2%), Phl p 6 (62.5%), Phl p 11 (50%), Phl p 7 (21%), and Phl p 12 (4%). We measured low concentrations of specific IgG₄ before AIT, which was most prominent for Phl p 4 (median, 0.07 ISU; IQR, 0.0-0.15 ISU) and Phl p 5 (median, 0.0 ISU; IQR, 0.0-0.15 ISU) in 17 and 13 patients, respectively.

Comparison of IgE and IgG₄ levels during SCIT up dosing with ImmunoCAP and ISAC

As found in previous studies,^{17,18} the mean concentration of allergen-specific IgE against the major allergen Phl p 5 (or Phl p 1 if no Phl p 5 was detected) increased from 23.0 to 48.8 kU/L ($P = .01$, $n = 18$) when measured by using ImmunoCAP (Fig 1, A). In contrast, ISAC measurement of specific IgE to the dominating grass pollen allergen Phl p 1 or Phl p 5 decreased significantly from a mean of 21.4 to 2.5 ISU ($P = .0002$, $n = 18$; Fig 1, B). There was a strong linear correlation between Phl p 1 and Phl p 5 IgE pretreatment measurements by using ImmunoCAP and ISAC ($P < .0001$, $n = 18$) but not after completed up dosing ($P = .20$, $n = 18$).

The mean concentration of corresponding IgG₄ increased when measured with either ImmunoCAP (0.1-5.2 mg/L, $P = .001$, $n = 18$; Fig 1, C) or ISAC (0.3-5.0 ISU IgG₄, $P = .0001$, $n = 18$; Fig 1, D). There was a strong linear correlation between IgG₄ levels toward Phl p 1 and Phl p 5 measurements both before treatment and after up dosing (both $P < .0001$, $n = 18$). IgE and IgG₄ immunoglobulin levels of the control group did not change.

Single-allergen ISAC measurements during SCIT

We expanded on this observation by assessing IgE and IgG₄ measurements against all 8 available grass pollen molecules found on ISAC (Fig 2). Allergen measurements without detectable IgE or IgG₄ at both time points in a given patient ($n = 42$) were censored. The measured concentration of IgE for all grass components decreased markedly from a median of 4.60 ISU (IQR, 1.66-13.98 ISU) to 2.14 ISU (IQR, 0.0-4.59 ISU; $P < .0001$; $n = 102$) during up dosing. At the same time, grass-specific IgG₄ concentrations increased from a median of 0.0 ISU (IQR, 0.0-0.11 ISU) to 0.83 ISU (IQR, 0.14-3.35 ISU; $P < .0001$; $n = 102$).

TABLE I. Baseline grass pollen-specific sensitizations

Subject		Major group 1/5 (ISU)		Other grass sensitizations (ISU)					
Patient ID	No. of component sensitizations	Phl p 1	Phl p 5	Phl p 2	Phl p 4	Phl p 6	Phl p 7	Phl p 11	Phl p 12
1	5	11.9	23.5	18.3		14.5		21.8	
2	3	2.6		5.1				3.7	
3	4	4.2	12.2	4.8	1.1				
4	6	4.7	28.3	3.7	1.8	3.9		9.0	
5	4	14.5	20.0		2.0	15.4			
6	2	1.4							
7	6	17.1	71.2	3.5	16.3	6.4	3.1		
8	6	28.3	51.3	38.8	6.3	7.6		10.0	
9	5	1.4	5.0	7.4		4.1		10.2	
10	6	7.9	16.1	3.4		4.7	2.4	4.5	
11	7	22.1	35.0	6.7	5.1	2.7	1.8	16.9	
12	6	17.5	57.8	4.8	2.3	3.7		4.5	
13	2	1.3	2.1						
14	5	2.8	15.5	11.1		3.0		11.8	
15	1	1.6							
16	5	7.7	31.4		1.8	5.6		16.6	
17	1		1.7						
18	6	10.8	28.5	19.1	1.6	9.2	14.0		
19	1		5.6						
20	6	2.8	8.1		1.8	1.9	1.4	6.2	
21	1	1.2							
22	5	25.0	21.0		7.0	7.4			0.7
23	4	40.0	35.0		10.0	16.0			
24	3	4.5		0.8	4.6				
Specific IgE (ISU), median (IQR)		4.6 (1.5-15.8)	15.8 (1.9-29.9)	2.1 (0-5.9)	1.4 (0-3.5)	3.4 (0-6.9)	0 (0-0)	1.9 (0-9.5)	0 (0-0)
No. of sensitized subjects (%)		22 (91.6)	19 (79.2)	13 (54.2)	13 (54.2)	15 (62.5)	5 (20.8)	12 (50)	1 (4.2)
Specific IgG ₄ (ISU), median (IQR)		0 (0-0.05)	0.07 (0-0.15)	0 (0-0.08)	0.07 (0-0.15)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
No. of subjects with detectable IgG ₄ (%)		6 (25)	13 (54.2)	7 (29.2)	17 (70.8)	2 (8.3)	3 (12.5)	3 (12.5)	2 (8.3)

Pretreatment levels of component-specific IgE (ISU): SCIT, patients 1-18; control, patients 19-24.

There were exceptions to this observation: in 4 treated patients we observed an increase in one grass component-specific IgE level (patient 5, Phl p 2; patient 10, Phl p 4; patient 6, Phl p 11; and patient 7, Phl p 12) from undetectable to moderately increased, with a median increase of 2 ISU. In one treated patient (patient 17) we found an increase in 2 IgE measurements (Phl p 1 and Phl p 2). These 5 patients did not induce IgG₄ to the above allergen molecules but mounted a clear IgG₄ response to Phl p 5 with a corresponding decrease in ISAC-measured Phl p 5 specific IgE levels.

Increase in allergen-specific IgG₄ levels correlates with decrease in allergen-specific IgE levels after up dosing

A major effect of SCIT is induction of IgG₄, which competes with cell-bound IgE and blocks IgE-induced allergic mechanisms.^{10,15,19} We observed a possible blocking activity of the IgG₄-containing sera on the detection of allergen-specific IgE at molecular resolution on ISAC; the concentration of IgG₄ for individual allergen molecules after up dosing correlated with the decrease in IgE concentration in ISU from the pretreatment visit to after up dosing (Spearman $\sigma = -0.56$, $P < .0001$, $n = 142$). When grouping samples according to IgG₄ concentration after up dosing (no detectable IgG₄, <0.03 ISU [$n = 56$]; lowest tertile, 0.04 - 0.50 ISU [$n = 29$]; middle tertile, 0.56 - 3.19 ISU [$n = 30$]; and highest tertile, 3.22 - 9.98 ISU [$n = 29$]), IgE measurement was significantly reduced in the middle tertile (median Δ IgE, -1.27 ISU; IQR, -4.68 to 2.93 ; $P = .037$), and this was more

pronounced in the highest tertile (Δ IgE, -12.00 ISU; IQR, -25.02 to -4.31 ; $P < .0001$; Fig 3).

The low concentration of pretreatment-specific IgG₄ measured did not interfere with detection of IgE on ISAC. The ratio between Phl p 5-specific IgE levels measured by using ISAC and CAP was not different between the group with and without detectable Phl p 5-specific IgG₄ levels (mean ratio in the presence of Phl p 5 IgG₄, 1.24 [0.94 - 1.53 , $n = 13$]; mean ratio without Phl p 5 IgG₄, 1.43 [0.54 - 2.33 , $n = 6$]; $P = .27$).

Pretreatment IgE concentration predicts IgG₄ concentration after the up dosing phase

As a corollary to this observation, we examined whether the pretreatment IgE concentration predicts the increase in IgG₄ concentration during the up dosing phase (Fig 4). We found a strong induction of IgG₄ response for 91.2% of molecules to which pretreatment component-specific IgE levels had been measured (Spearman $\sigma = 0.83$, $P < .0001$). Specific IgE to grass allergens was less than the detection level in 64 (44.4%) measurements. In these samples we only observed minor changes in the IgG₄ concentration after the up dosing phase (median change, 0.13 ; IQR, 0.06 - 0.24 ; $n = 14$). The 57 measurements with a moderate to high increased pretreatment level of IgE ($1 < x < 15$ ISU, 39.6%) associated with a median increase of 0.87 ISU (IQR, 0.2 - 3.06 ISU; $P < .001$) IgG₄ after up dosing. In all cases with a very high concentration of pretreatment IgE (>15 ISU, $n = 23$, 16.0%), we found an induction of IgG₄ with a median increase of 4.12 ISU (IQR, 1.52 - 8.38 ; $P < .001$).

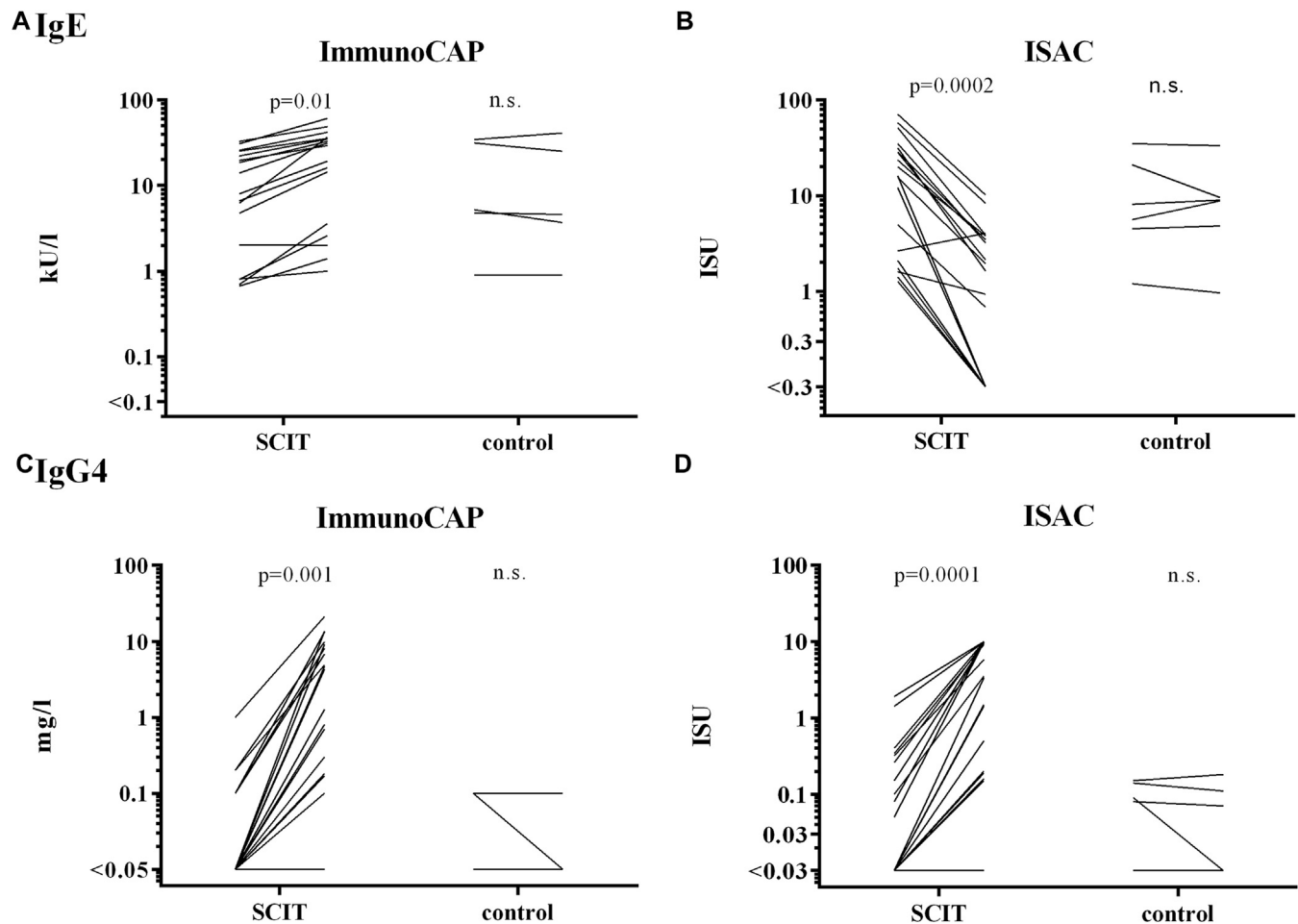


FIG 1. Measurements of levels of IgE and IgG₄ to the major grass pollen components (Phl p 1 or Phl p 5) by using ImmunoCAP and ISAC. Pretreatment levels of specific IgE are comparable, as measured by using both methods. After up dosing of SCIT, IgE ImmunoCAP measurements increased (**A**), whereas IgE ISAC measurements decreased (**B**). IgG₄ measurements were comparable, showing a marked increase after up dosing (**C** and **D**). Measurements of less than the detection limit are plotted as 0.1 kU/L and 0.1 ISU (IgE) and 0.01 mg/L and 0.01 ISU (IgG₄) in all figures, respectively. Treatment, n = 18; control, n = 6. *n.s.*, Not significant.

IgG₄ was not induced in 7 (8.8%) of the instances in which low to moderate IgE concentrations ($0.3 < x < 15$ ISU) were measurable at baseline. Patients 14 and 15 with pretreatment IgE to Phl p 1 and patients 7 and 11 with IgE to Phl p 7 did not induce measurable relevant IgG₄. Patient 10, with a significant concentration of pretreatment-specific IgE against Phl p 1, Phl p 2, and Phl p 7, did not induce IgG₄ to these allergens after up dosing. This subject mounted only a weak IgG₄ response to Phl p 5 as well. Despite these findings, the 5 patients experienced symptom relief comparable with that in the other treated patients.

Correlates in cellular tests and clinical outcome

Because changes in allergen-specific immunoglobulin concentrations only are one of the complex changes occurring during AIT that lead to allergen tolerance, we tested whether these immunoglobulin changes correlated with other more functional immunologic changes as FAB and basophil sensitivity and improvement in clinical outcome.

Consistent with the theory that antigen presentation through IgE²⁰ facilitates immune response at much lower antigen

concentrations (FAP),²¹ we found a negative correlation between pretreatment grass pollen-specific IgE and the relative inhibition of FAB after up dosing (Spearman $\sigma = -0.43$, $n = 23$, $P = .04$).¹⁵ This can be explained mainly by allergen-specific induction of IgG₄: we found a strong correlation between FAB inhibition and induced IgG₄ levels after up dosing (Spearman $\sigma = 0.59$, $n = 24$, $P = .0027$).

Basophil sensitivity¹⁵ correlated inversely with the ratio between the sum of all grass pollen-specific ISAC IgE/total IgE levels both at baseline (Spearman $\sigma = -0.56$, $P = .005$) and after up dosing (Spearman $\sigma = -0.74$, $P < .0001$) and to the ratio of the sum of all ISAC grass pollen-specific IgE/IgG₄ levels after up dosing (Spearman $\sigma = -0.69$, $P = .0008$).

Among clinical outcomes, the maximum symptom score of the grass pollen season after up dosing correlated to the postup dosing sum of grass-specific IgE levels (slope, 0.14; 95% CI, 0.07-0.21; $P = .001$), IgG₄ levels (slope, -0.19 ; 95% CI, -0.34 to -0.03 ; $P = .019$), and basophil sensitivity (slope, 3.02; 95% CI, 0.38-5.65; $P = .028$) but not to FAB results (slope, 0.01; 95% CI, -0.06 to 0.07 ; $P = .85$) after up dosing in a multiple regression model with a cumulative correlation coefficient of 0.78.

All grass components

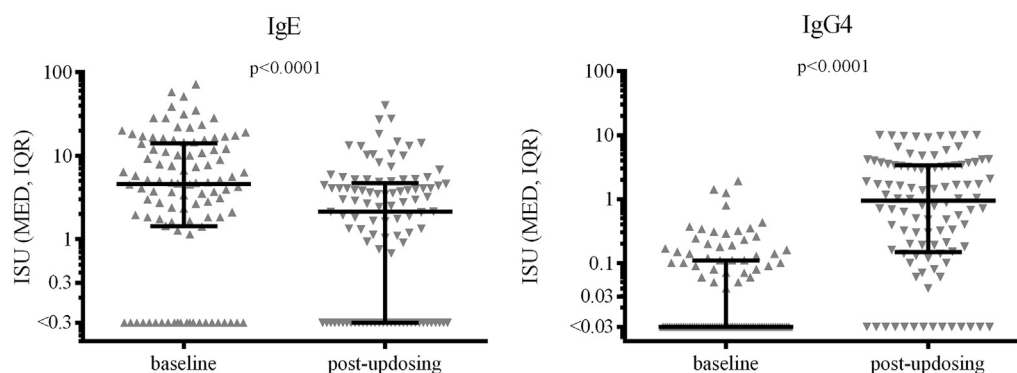


FIG 2. ISAC pretreatment and postupdosing measurements of all grass component-specific IgE and IgG₄ levels. IgE levels of all grass components were clearly reduced during the updosing phase of SCIT, whereas immunotherapy induces a strong IgG₄ response. Measurements with no detectable IgE or IgG₄ at any time point were censored ($n = 42$), resulting in 102 measurements displayed.

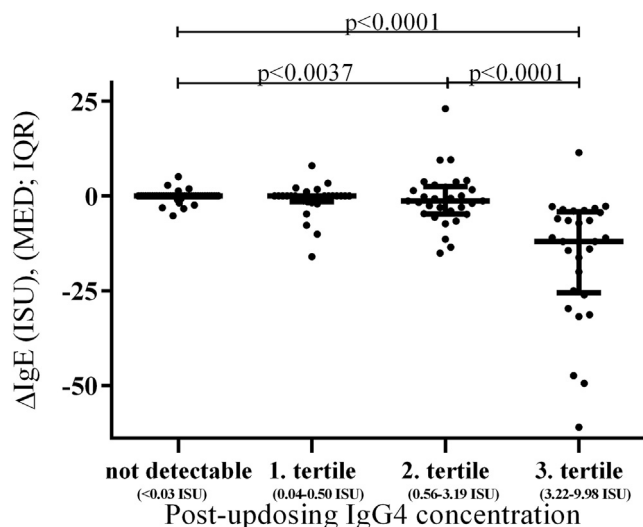
IgG₄ suppresses IgE measurements

FIG 3. Induced IgG₄ suppresses ISAC IgE measurements. All grass pollen component-specific IgG₄ measurements were divided into 4 groups. Group 1 had no detectable IgG₄ after updosing ($n = 56$). The remaining 88 measurements were distributed into 3 tertiles ($n = 29$, $n = 29$, and $n = 30$) and plotted against the changes in component-specific IgE levels occurring during updosing. A higher postupdosing IgG₄ level clearly suppressed IgE after updosing.

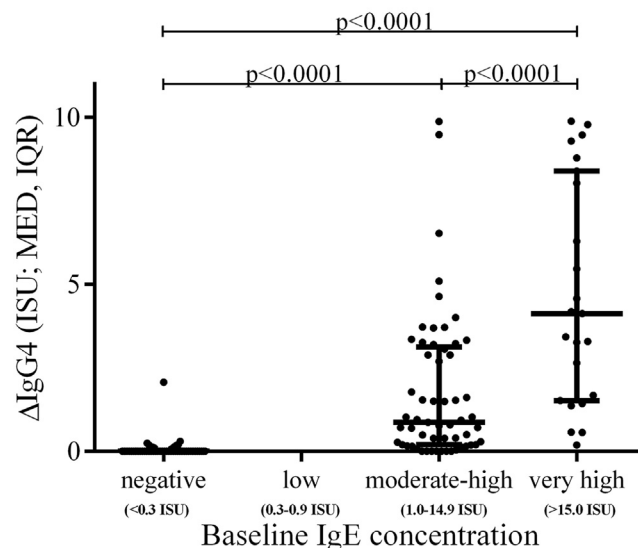
Pretreatment IgE induces IgG₄

FIG 4. Pretreatment IgE predicts IgG₄ induction. Induction of IgG₄ depended on the concentration of pretreatment component-specific IgE. Without a detectable level of component-specific IgE at baseline, no IgG₄ was produced during updosing of SCIT ($n = 64$), a moderate to high pretreatment IgE concentration resulted in a moderate increase in IgG₄ ($n = 37$), and a very high level of pretreatment component-specific IgE led to a strong induction of IgG₄ ($n = 23$).

Reproducibility of ISAC measurements

Measurements of grass component-specific IgE did not differ between the 2 time points in the control group (median change, 0.0 ISU; IQR, 0.0-0.0 ISU; $n = 48$). The concentration of IgE to nongrass components on ISAC did not change during updosing with grass allergen (median at baseline, 0.0 ISU [IQR, 0.0-2.07 ISU]; median after updosing, 0.0 ISU [IQR, 0.0-2.57 ISU]; $n = 423$, $P = .23$, Fig 5). When comparing the repeated ISAC measurements of non-grass-specific IgE ($n = 95$ per patient), we found sporadic measurements ($n = 49$, 2.15%) with a median increase of 2.08 ISU, leading to a change by at least 1 ISU class, mostly from undetectable to a moderate level. Only one

increasing measurement (2.0%) could be explained by cross-reactivity to the profilin Phl p 12. Most increasing measurements ($n = 41$, 84%) were related to birch pollen cross-reactivity and could probably be explained by natural allergen exposure during the birch pollen season between the 2 measurements. Fifty (2.19%) measurements decreased by a median of 1.43 ISU, resulting in a lower ISAC class, mostly from moderate to undetectable. Twelve (24%) of these decreasing measurements were also related to birch pollen cross-reactivity. IgG₄ levels specific for nongrass components increased from a median of 0.32 ISU (IQR, 0.0-1.13 ISU) to 0.4 ISU (IQR, 0.0-1.40 ISU; $n = 423$, $P < .0001$, Fig 5). This minimal increase was statistically

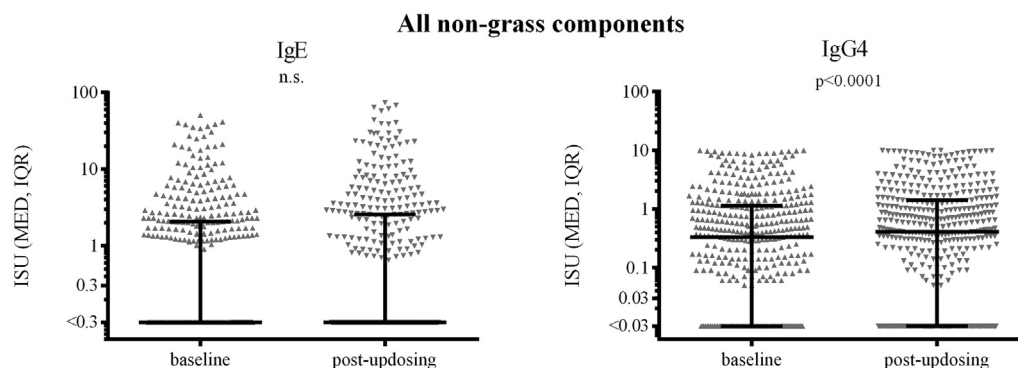


FIG 5. Comparison of all non-grass-specific ISAC IgE and IgG₄ measurements. Non-grass-specific IgE concentrations remained unchanged during the updosing phase of SCIT. IgG₄ measurements increased slightly ($n = 423$). These increases were mostly related to birch pollen cross-reacting epitopes. *n.s.*, Not significant.

significant and could be explained by IgG₄ measurements related to birch pollen and allergens cross-reactive to birch pollen. Removing measurements related to birch pollen resulted in a median non-grass-specific IgG₄ level of 0.44 ISU (IQR, 0.06-1.65 ISU) before and 0.43 ISU (IQR, 0.09-2.02 ISU; $P = .12$; $n = 262$) after updosing.

DISCUSSION

In this study we investigated early changes in allergen component-specific IgE and IgG₄ concentrations during the updosing phase of SCIT in patients with grass pollen allergy. We found that pre-existing IgE predicts increases in IgG₄ levels to individual allergen molecules after updosing and that this IgG₄ measurement was a good marker for immunoglobulin that competes with IgE for binding to the limited amount of allergen present on ISAC.

Baseline measurements

The studied population had a slightly higher prevalence of IgE sensitization to Phl p 1 and Phl p 5 (Table 1) than earlier population-based studies.²² This can be explained by the selection of the study subjects because only subjects with a symptom burden sufficiently high to be eligible for AIT and with a baseline basophil reactivity to grass pollen extract of greater than 40% were included. However, the prevalence of component-specific sensitizations was comparable with those reported at <http://www.allergen.org>. Compared with a study in 101 patients with grass pollen allergy from Germany²³ and a Spanish study including 139 patients with grass allergy,²⁴ we found a higher level of Phl p 5-specific IgE and a lower level of Phl p 4-specific IgE, which might be explained by regional differences in exposure patterns. We did not find a relation between disease duration and sensitization pattern (data not shown) described in a recent pediatric study.²⁵

ISAC measurement of IgE to treatment allergens is suppressed by AIT

As in a previous study,²⁶ we found comparable pretreatment IgE concentrations with both ImmunoCAP and ISAC. SCIT upregulated allergen-specific IgE levels^{17,18,27} when measured with the ImmunoCAP method where allergen is in excess. In

contrast, signals in the ISAC measurement of allergen-specific IgE were significantly reduced by SCIT. A consequence of this reduction was a loss of detection (<0.3 ISU) of half of the pretreatment component-specific IgE measurements recorded with ISAC. This reduction in IgE-related signals assessed by using ISAC might be a useful companion diagnostic to monitor the efficacy of SCIT. The only other study exploring the effect of AIT on CRD by using ISAC dealt with sublingual immunotherapy of patients with allergy to house dust mites. There was no effect of a 1-year course of sublingual immunotherapy on IgE concentration but a moderate increase in IgG₄ levels.²⁸

IgG₄ induced by SCIT suppresses ISAC IgE measurements

We investigated the origin of the suppression of IgE measurements on ISAC by measuring IgG₄ levels with ISAC because induction of IgG₄ is a useful marker for the immunologic effect of AIT.^{17,18,27} We found a significant inverse correlation when reaching maintenance between IgG₄ levels induced by AIT and the apparent reduction of the IgE signal, suggesting that an increase of greater than 0.56 ISU of IgG₄ (second and third tertiles) leads to a measurable decrease by competing with IgE for epitopes of the limited amount of allergen spotted on the chip. This is probably an underestimate of the suppressive effect of IgG₄ because the concentration of IgE to the major allergens Phl p 1 and Phl p 5 in simultaneous ImmunoCAP measurements increased significantly. Similar results were obtained²⁹ when component-specific IgG antibody spiked into serum dose-dependently reduced the apparent IgE concentration on the new MeDALL chip. At a concentration of 2.5 ISU of IgE, a thousand-fold excess of IgG blocked IgE binding completely. The effect of IgG competition increased with IgE concentration for antibodies with identical allergen-binding affinity. In the present study IgE measurement for Phl p 1 and Phl p 5 in ImmunoCAP doubled, and pretreatment measurements with ISAC were reduced to approximately 10% after updosing. That would correspond to an 18-fold reduction of ISAC measurement by IgG similar to what was found with a 100-fold excess of IgG by using the MeDALL chip.²⁹ The IgG₄ concentration increased at least 41-fold from less than the detection limit of 0.02 ISU to a median of 0.83 ISU, which is in the expected order of magnitude

considering that other immunoglobulin species also compete with IgE. The significance of specific IgG₄ induction is further enhanced by good correlation of the IgE/IgG₄ ratio with basophil sensitivity described in a previous study of the effect of AIT on basophil sensitivity.³⁰ We have previously reported that basophil sensitivity at 3 weeks predicts clinical symptoms during the ensuing season,¹⁵ and this might indicate that early ISAC measurements can be used as companion diagnostics.

Evidence for a link between pretreatment specific IgE and IgG₄ after up dosing

Subsequently, we investigated whether specific IgG₄ was produced independently of pretreatment-specific IgE because the generated specific IgG₄ appeared to suppress the existing specific IgE molecules. In nearly all instances, we measured specific IgG₄ levels after up dosing only when we had measured the same specificity for IgE at baseline. We only found 5 among 144 measurements for which we had no pretreatment-specific IgE but did measure the corresponding specific IgG₄ levels when reaching maintenance. The major finding of this study is that pretreatment-specific IgE might be a prerequisite for induction of a specific IgG₄ response during SCIT. This is supported by findings in a 12-week study of patients with ragweed allergy treated with a course of rush immunotherapy with and without anti-IgE treatment, in which the IgG₄ response was weaker and developed more slowly when immunotherapy was combined with anti-IgE treatment that removed IgE.³¹

Mechanism linking pretreatment IgE and IgG₄ after up dosing

IgE can play an important role in the immune-modulating process occurring during SCIT up dosing. This function of IgE might be facilitated antigen presentation (FAP); CD23 on B cells or FcεRI on dendritic cells captures allergen and facilitates its efficient presentation at markedly reduced concentrations. The current data are a strong *in vivo* observation consistent with FAP in a relevant clinical context and are the first observation that IgG₄ concentrations after up dosing correlate with pretreatment IgE concentrations, directly linking the well-established AIT-induced modulator IgG₄ to FAP.³² The first demonstration of FAP in human subjects was that T cells proliferated at 100 to 1000 times lower allergen concentrations when facilitated by allergen-specific IgE³³ *in vitro*. The IgE-mediated T-cell activation (FAP) has later been shown to correlate closely with binding of allergen-IgE complexes to the surfaces of B cells (FAB) for birch,^{34,35} and this mechanism has been demonstrated for other allergens, such as grass³⁶ and peanut.²⁰

Binding of individual allergen molecules by specific IgE might also be important for presenting that allergen molecule to allergen-specific B cells, resulting in specific IgG₄ production to the same molecule. The binding measured by using FAB and the T-cell activation measured by using FAP might both contribute to the induction of IgG₄ and other non-IgE antibodies specific to the relevant allergens, and subsequently, these non-IgE antibodies inhibit both mechanisms.

The correlation of inhibition of FAB with the inverse of pretreatment IgE and with induced IgG₄ and the observation that IgG₄ is induced only where pretreatment IgE exists suggest

that FAP supports an early induction of protective IgG₄ during the up dosing phase of AIT.

The situation at the ISAC surface is a complex dynamic competition for physiologically relevant amounts of allergen between IgG₄ and IgE,²⁹ which might reflect the *in vivo* situation in which free specific immunoglobulin creates complexes with allergen in competition with IgE presented on effector cells. This might explain the correlation between clinical symptoms and concentration of component-specific IgE and IgG₄ and effector cell sensitivity we found in a multiple regression model.

ISAC is a reproducible assay

The ISAC assay has been shown to have good reproducibility and repeatability.³⁷ A few ISAC measurements (2%) changed status during the 2 measurements 16 weeks apart, reinforcing the word of caution that IgE results always should be interpreted within a clinical context. Most increases could be explained by the intervening birch pollen season.

Five patients had possible neosensitizations to grass pollen components. The clinical outcome of these 5 patients was comparable with that of the other treated patients. It is unclear whether these measurements represent a real neosensitization or whether the component-specific IgE levels merely increased from less than the detection limit. Earlier studies suggesting that neosensitization occurs during SCIT^{38,39} have not been confirmed by others.^{40,41}

Limitations of our study

Only 24 subjects were studied; a bigger study might be needed to confirm our findings. Allergen challenge outcomes had been performed in 10-fold dilution steps. Clinical response would perhaps correlate better with biomarkers if the intervals had been shorter or analyzed with a more refined model.^{42,43} This study addresses the up dosing phase of SCIT; different mechanisms can operate during the maintenance phase and after AIT has ceased. Larger studies addressing changes in component-specific IgE levels before and after IgG depletion might confirm the blocking effect of IgG₄ on IgE detection on ISAC.

General considerations: Further implications

It will be important to determine whether pretreatment IgE is directly involved in induction of IgG₄ with the help of IgE-FAP. Several novel vaccination programs are based on hypoallergenic molecules^{44,45} for vaccination that are designed not to bind IgE and might not be presented through IgE. In future studies, it would be interesting to investigate whether the strong inductive effect of pretreatment IgE on the production of IgG₄ during immunotherapy can be explained by pre-existing B-cell clones having a molecule-specific antibody repertoire or whether IgE-mediated facilitated allergen presentation induces this response by ensuring allergen uptake on the component-specific level to B cells, resulting in the production of IgG₄ specific to all relevant allergens.

Nonmodified allergen extracts can contain variable amounts of or even lack important allergen components,¹² and there might be a risk of an incomplete treatment effect in patients allergic to components lacking or only poorly represented in the natural allergen extract. Treatment with recombinant allergen component

vaccines covering all the patients' sensitizations have been suggested to be an effective⁴⁶ and safe⁴⁷ method of AIT and offer the potential of individualizing AIT after the pretreatment CRD profile of the individual patient. However, we find that the use of a well-standardized natural extract in SCIT fully matches patients' sensitizations.

Conclusion

We found that pretreatment allergen component-specific IgE predicted the induction of IgG₄ to allergen components, resulting in a strong correlation between the pretreatment IgE level and IgG₄ concentrations after updosing. IgE-induced IgG₄ antibodies seem to compete for allergen binding on ISAC, leading to suppression of the IgE-related signal on this chip. They correlate to FAB, basophil sensitivity, and symptom severity. Measurement of IgE levels on ISAC before and after updosing of SCIT can be used as an early biomarker of AIT responses.

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Clinical implications: IgE and IgG₄ sensitizations measured at molecular resolution before and after updosing of SCIT might be useful to monitor the early treatment effect. Treatment with allergen extract induces IgG₄ matching the pretreatment component-specific IgE.

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