

Heterogeneity of antibody responses among clinical responders during grass pollen sublingual immunotherapy

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Summary

Background During allergen-specific sublingual immunotherapy (SLIT), the relevance of changes in specific IgE and IgG antibody titres to treatment efficacy remains to be evaluated at an individual patient level.

Objective To investigate whether antibody responses can be used as biomarkers for SLIT efficacy.

Methods Comprehensive quantitative, qualitative and functional analyses of allergen-specific IgA, IgE, IgG1–4 and IgM responses were performed using purified Phl p 1 to 12 allergens in sera, saliva and nasal secretions from 82 grass pollen allergic patients. These patients were enrolled in a randomized, double-blind placebo-controlled study and assessed in an allergen challenge chamber (ClinicalTrials.gov NCT00619827). Antibody responses were monitored in parallel to clinical responses before and after daily sublingual treatment for 4 months with either a grass pollen or a placebo tablet.

Results A significant mean improvement (i.e. 33–40.6%) in rhinoconjunctivitis total symptom scores was observed in SLIT recipients, irrespective of their baseline patterns of IgE sensitization (i.e. narrow, intermediate, broad) to grass pollen allergens. SLIT did not induce any *de novo* IgE sensitization. Clinical responders encompassed both immunoreactive patients who exhibited strong increases in titres, affinity and/or blocking activity of grass-pollen-specific IgGs (representing 17% of treated patients), as well as patients with no detectable antibody responses distinguishing them from the placebo group. No significant changes were detected in antibody titres in saliva and nasal washes, even in clinical responders.

Conclusions and Clinical Relevance Sublingual immunotherapy with a grass pollen tablet is efficacious irrespective of the patients' baseline sensitization to either single or multiple grass pollen allergens. Seric IgG responses may contribute to SLIT-induced clinical tolerance in a fraction (i.e. 17%) of patients, but additional immune mechanisms are involved in most patients. Consequently, antibody responses cannot be used as a marker of SLIT efficacy at an individual patient level.

Keywords allergen, antibody, grass pollen, neosensitization, sublingual immunotherapy

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Introduction

The clinical efficacy of grass pollen sublingual immunotherapy (SLIT) has been established in large cohorts of patients [1–3]. Despite evidence that successful immunotherapy induces (i) an upregulation of specific IgG4s, (ii) the polarization of CD4⁺ T cell responses

towards a Th1/T regulatory pattern [4–7] and (iii) the expression of regulatory dendritic cell markers detectable in peripheral blood [8], there is as of today no established surrogate biomarker for SLIT efficacy [9, 10].

In this regard, the follow-up of changes in specific antibody responses elicited during immunotherapy is

often considered as a fast and convenient way to confirm the impact of immunotherapy on the patients' immune system. Classically, levels of allergen-specific IgEs are initially up-regulated prior to decreasing to baseline following approximately 1 year of treatment [11–14]. In contrast, IgG4s are systematically induced within weeks and remain sustained throughout the treatment period [3, 13, 15–17]. Recently, specific functions of IgGs more than seric levels were found to correlate with clinical benefit during immunotherapy, with the hypothesis that high affinity IgGs can better compete with IgEs for binding to allergen(s) and thus act as IgE-blocking factors [18, 19]. Specifically, IgE-mediated processes such as basophil histamine release [20], allergen binding to B cells and allergen presentation to T cells [21], are all inhibited by such blocking IgGs.

Beyond those observations, many important questions remain unanswered. No clear correlation between antibody responses and clinical benefit has been yet established for individual treated patients. Furthermore, the efficacy of immunotherapy in patients exhibiting distinct patterns of IgE sensitization (e.g. to major and/or minor allergens) remains debated, with the notion that natural extracts may not contain all needed allergens [22]. Alternatively, IgE neosensitization to allergens present in the vaccine may occur during specific immunotherapy, although in this regard, the risk appears higher with the subcutaneous route when compared with sublingual administration [12, 23–26].

To address these questions, we performed an extensive analysis of antibody responses in sera and mucosal fluids from 82 grass pollen allergic patients receiving daily for 4 months a sublingual tablet containing either a 5-grass-pollen extract or a placebo [27]. In this double-blind, placebo-controlled study performed outside of the pollen season, patients were exposed before, during and after treatment to grass pollen in an allergen challenge chamber (ACC). Patients with a significant reduction in the average rhinoconjunctivitis total symptom score (ARTSS) at the end of treatment were classified as clinical responders. Using this cohort, we demonstrate herein that SLIT is equally efficacious in patients with either narrow or broad patterns of IgE sensitization, without any induction of *de novo* sensitization. We also show the absence of correlation between clinical improvement and antibody responses, with a clear heterogeneity in the latter observed among clinical responders.

Materials and methods

Design of the VO56.07A chamber exposure study

Details of this double-blind, placebo-controlled clinical study (ClinicalTrials.gov NCT00619827) have been

published elsewhere [27]. Briefly, for 4 months starting from the randomization visit (V3), 82 grass pollen allergic patients received sublingually one daily tablet containing either a placebo or a 5-grass-pollen extract (Stallergenes, Antony, France) [28]. Patients were exposed outside of the pollen season to grass pollens in an ACC before (baseline challenge), as well as after 1 week and 1, 2 and 4 months of treatment. Patients recorded their rhinoconjunctivitis total symptom score (nasal and eye symptoms) during each ACC session, which served as a treatment efficacy variable [27]. Samples of blood, nasal washes and saliva collected from each patient before (V3) and after 4 months (V7) of treatment were analysed. To collect nasal washes, each nostril was rinsed with 3 mL of 0.9% NaCl solution. After adding a protease inhibitor solution (Complete, EDTA-free protease inhibitors; Roche, Meylan, France), samples were centrifuged and supernatants frozen at -80°C . All samples were blind-coded to the operators.

Measurements of antibody titres in sera, saliva and nasal secretions

Titres of IgA, IgE, IgG and IgG4 specific for *Phleum pratense* allergens (i.e. recombinant Phl p 1, 2, 5b, 6, 7, 11, 12 and natural Phl p 4) or a 5-grass-pollen extract (Stallergenes) were determined using the ImmunoCAP system (Thermo Fisher Scientific, Uppsala, Sweden). IgA1, IgA2, IgE, IgG1, IgG2, IgG3, IgG4 and IgM antibodies reactive with the 5-grass-pollen extract were measured in plasma samples by ELISA as described elsewhere [29]. The reactivity of antibodies (IgEs, IgG3s and IgG4s) was assessed by immunoblotting after separation of proteins from a *Dactylis glomerata* pollen extract by isoelectrofocalization (pH 3–10) on nitrocellulose membranes as described elsewhere [30].

IgE-blocking assay

The IgE-blocking activity was measured in plasma samples using a cytometric bead array human IgE Flex set (BD Biosciences, San Diego, CA, USA) with a method adapted from the study by Würtzen et al. [15]. Briefly, two aliquots of each plasma sample were incubated in parallel with beads coated with anti-IgE antibodies. Following capture of the IgEs, beads from one aliquot were washed to remove potential competing components. A biotinylated 5-grass-pollen extract (40 ng/mL) was added to both washed and unwashed beads to allow IgE binding. After washing, both aliquots were allowed to react with streptavidin-PE (BD Biosciences), further washed and analysed by FACS (FC500; Beckman Coulter, Brea, CA, USA). An IgE-blocking factor was calculated based on the ratio between mean fluorescence

intensities (MFI) measured for the two aliquots as: 1-MFI (unwashed beads)/MFI (washed beads).

IgE-facilitated allergen binding assay

IgE-facilitated allergen binding (FAB) assay was adapted from the study by Shamji *et al.* [21]. Briefly, a pool of sera from grass pollen allergic patients (indicator pool, containing 327 kU/L of grass-pollen-specific IgEs) was pre-incubated at 37°C for 1 h in the presence or absence of an equal volume of serum from a study patient, together with 25 ng protein/mL of a 5-grass-pollen extract to allow formation of allergen-IgE complexes. Subsequently, 2×10^5 CD23⁺ B cells (RPMI 8866; Sigma-Aldrich, St. Louis, MO, USA) were added and incubated for 1 h at 4°C. After washing, allergen-IgE complexes bound to cells were detected with a polyclonal fluorescein isothiocyanate - labelled anti-IgE antibody (Invitrogen, Carlsbad, CA, USA) by flow cytometry (FC500 apparatus). Relative binding of IgEs to B cells was calculated as follows: (% IgE-FAB using the indicator pool in presence of the patient's serum/% IgE-FAB using the indicator pool only) $\times$ 100.

Measurement of antibody affinities for grass pollen allergens by surface plasmon resonance

Surface plasmon resonance (SPR) was used to analyse the interactions between seric IgA, IgE, IgG3, IgG4 and IgM antibodies and purified Dac g 1 or Dac g 5 allergens [31]. Immunoglobulins were purified from individual patients' sera on 96-well plates (Greiner, Frickenhausen, Germany) coated with biotinylated anti-isotype antibodies (Invitrogen) using Streptavidin HP MultiTrap system (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. Purity of each antibody isotype was confirmed by a dot-blot analysis. Spot intensities were integrated to normalize the amounts injected for SPR analysis. Binding experiments were performed on a Biacore 3000 (GE Healthcare) with a constant flow rate of 30 μ L/min of HBS-EP running buffer (10 mM Hepes, 350 mM NaCl, 0.005% surfactant P20, 3 mM EDTA, 1% DMSO). Binding experiments with purified antibodies were performed in duplicate on a CM5 sensorchip (Biacore; GE Healthcare) functionalized by 2000 RU (response unit) Dac g 1 or Dac g 5 purified allergens.

Statistical analyses

Data are represented using Tukey box plots summarizing median, upper and lower quartiles. Outliers (i.e. values inferior or superior by 1.5-fold to lower and upper quartiles, respectively) were plotted as dots. A Wilcoxon matched-pair signed rank test was used to assess

changes in biological parameters between V3 (randomization) and V7 (end of treatment) within groups, and a Mann-Whitney test was implemented to compare results obtained in patients from active or placebo groups at V7. The Kruskal-Wallis test was applied to investigate the link between clinical efficacy and patterns of IgE sensitization, and Spearman tests were used to study correlations.

Results

Summary of clinical efficacy analysis

The clinical outcomes of this trial have been reported previously [27]. Briefly, a 29.3% mean reduction in the ARTSS was observed in patients receiving the active treatment vs. placebo after 4 months of SLIT, demonstrating the efficacy of the grass pollen sublingual tablets on allergic symptoms (with a statistical difference between ARTSS of active and placebo groups, $P = 0.0003$). As reported elsewhere, individual clinical responders and non-responders were identified both within the active and placebo groups, using a 43.9% decrease in the ARTSS after treatment as a threshold limit, corresponding to the median percentage of ARTSS improvement in the active group [8].

Sublingual immunotherapy is efficacious irrespective of the patients' patterns of IgE sensitization

At baseline, all patients were found to be sensitized (IgE levels > 0.35 kU/L) to group 1 and group 4 grass pollen allergens, except for one patient monosensitized to Phl p 4. Three different patterns of sensitization were defined in the entire study population based on the range of IgE reactivity to *P. pratense* allergens (Phl p 1, 2, 4, 5b, 6, 7, 11 or 12) at baseline, including (i) pattern I (narrow, with IgEs reactive to 1–3 allergens, $n = 24$), (ii) pattern II (intermediate, with IgEs reactive to 4–5 allergens, $n = 32$) and (iii) pattern III (broad, with IgEs reactive to 6–8 allergens, $n = 26$) (Fig. 1a). These patterns were confirmed by a qualitative analysis of IgE reactivity to proteins from a *D. glomerata* pollen extract, after separation by immunoblotting after isoelectric focusing (IEF) in a wide pH gradient and immunoblotting (Fig 1b). Data from four representative patients (2 from each of the placebo and active groups) are shown with patients 1 and 3 exhibiting a narrow pattern of sensitization, whereas patients 2 and 4 had a broader pattern. Importantly, there was no relationship between those patterns of IgE sensitization and clinical efficacy, because no significant difference was observed in mean percentages of ARTSS improvement (i.e. 37.3%, 33.0% and 40.6%; $P > 0.05$, Kruskal-Wallis test) between patients from the active group exhibiting

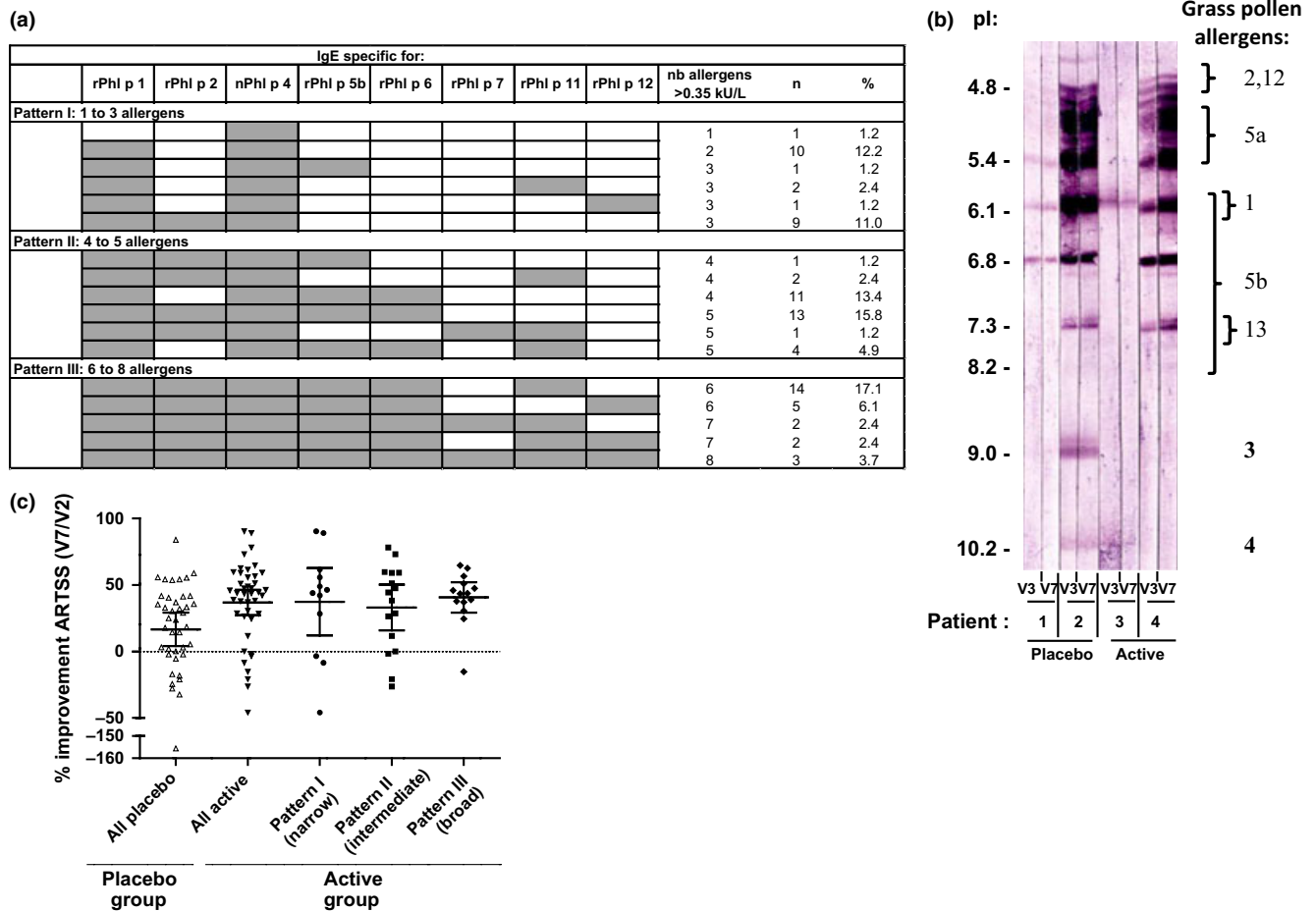


Fig. 1. Clinical efficacy of sublingual immunotherapy (SLIT) in grass pollen allergic patients with various patterns of IgE sensitization. IgEs specific for purified *Phleum pratense* allergens Phl p 1, 2, 4, 5b, 6, 7, 11, 12 or for a 5-grass-pollen extract were measured by ImmunoCAP in sera from 82 patients before treatment. (a) three distinct patterns of IgE sensitization were determined based upon number of allergens to which IgEs were reactive including pattern I (one to three allergens), pattern II (four to five allergens) and pattern III (six to eight allergens). Grey squares correspond to a positive signal (IgE level > 0.35 kU/L). Numbers of patients (*n*) with a given pattern of sensitization and corresponding percentages relative to the whole cohort are presented on the right side. (b) The reactivity of seric IgEs to proteins from a *Dactylis glomerata* pollen extract was analysed by immunoblotting after isoelectric focusing (IEF) separation using a wide pH gradient. Results obtained with sera from representative patients receiving either placebo (patients 1 and 2) or active treatment (patients 3 and 4) are shown, before (V3) and after a 4-month SLIT (V7). (c) Improvement in average rhinitis total symptom score (mean average rhinoconjunctivitis total symptom score improvement $\pm$ SEM) relative to baseline values is shown for subsets of patients in relation to their patterns of IgE sensitization.

patterns I, II or III, respectively, to be compared with a 16.6% decrease in ARTSS in the placebo group (Fig. 1c). Moreover, no correlation was found between levels of IgE specific for either one of *P. pratense* allergens before treatment and clinical responses (not shown).

Serum IgE, IgG4 and IgG responses induced during treatment

Significant increases in grass-pollen-specific IgEs (1.6–4.3-fold) and IgG4s (1.5–4.9-fold) were observed during SLIT in the active group for all allergens except Phl p 7 for IgEs and Phl p 7 and Phl p 12 for IgG4s, for which

no significant variations were noticed (Fig. 2). As expected, no major changes occurred in patients receiving placebo. After treatment, levels of IgEs reactive to Phl p 1, and IgG4s reactive to Phl p 1, 5b or the 5-grass-pollen extract were significantly higher in the active group when compared with the placebo group (V7) ($P < 0.05$), confirming that these changes are related to immunotherapy. Similar differences were observed between the active and placebo groups in levels of total IgGs, with most prominent differences seen for IgGs reactive to Phl p 5b or 5-grass-pollen extract (Fig. 2c). No major changes were detected by ELISA for grass-pollen-specific IgA1s, IgA2s, IgG1s, IgG2s, IgG3s and IgMs (not shown).

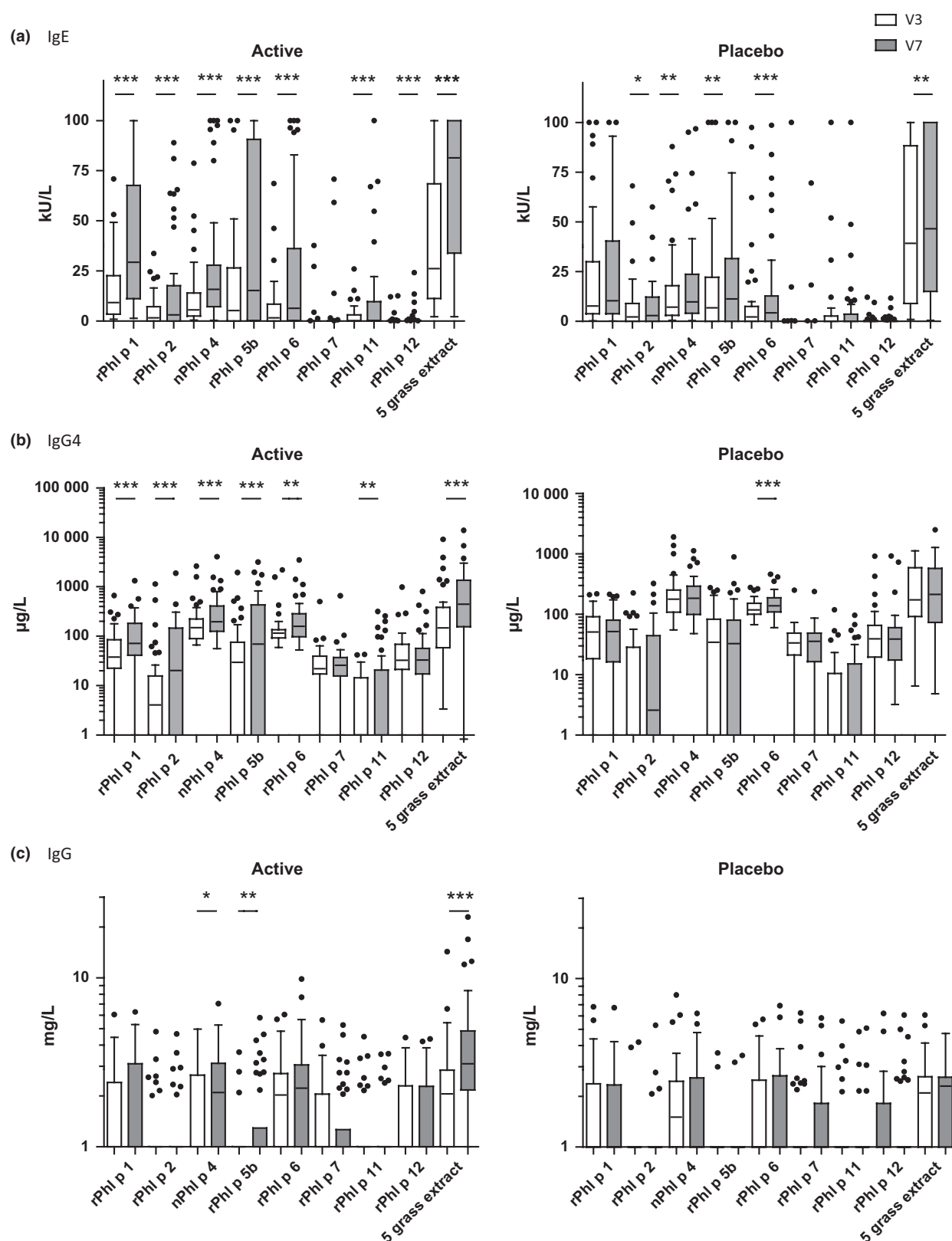


Fig. 2. IgE, IgG4 and IgG responses to grass pollen allergens during sublingual immunotherapy. IgEs (a), IgG4s (b) or total IgGs (c) specific for grass pollen allergens (as indicated) were quantified by ImmunoCAP in sera collected before (V3, white boxes) and after (V7, grey boxes) treatment with either active ($n = 42$) or placebo ($n = 40$) tablets. In the Tukey box plots shown, outliers (values inferior or superior by 1.5-fold to lower and upper quartiles, respectively) are plotted as dots (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Wilcoxon matched-pairs signed rank test).

Importantly, in patients who had no detectable IgEs specific for a given allergen before treatment, no induction was observed during SLIT, whereas, as expected, pre-existing IgE titres were boosted in sensitized patients from the active, but not the placebo group (Fig. 3a). The absence of IgE neosensitization was further confirmed on immunoblots, as shown in Fig. 1b, comparing profiles obtained before (V3) and after (V7) treatment for two representative patients. Similarly, SLIT boosted pre-existing IgG4 responses in patients from the active group, but did not elicit IgG4s with new specificities for most (i.e. > 75%) patients (Fig. 3b).

Limited antibody responses induced by active treatment at mucosal surfaces

Saliva and nasal washes collected before and after treatment were used to evaluate mucosal antibody responses induced by SLIT. IgA, IgE and IgG4 antibodies specific for Phl p 1, Phl p 5b or 5-grass-pollen extract were quantified by ImmunoCAP. Surprisingly, whereas a readily detectable increase in seric IgEs was observed in patients receiving active tablets (Fig. 2a), no such changes occurred in saliva or nasal washes (Figs 4a and b, left panels). A minor (≤ 2 -fold) upregulation was occasionally observed in grass-pollen-specific

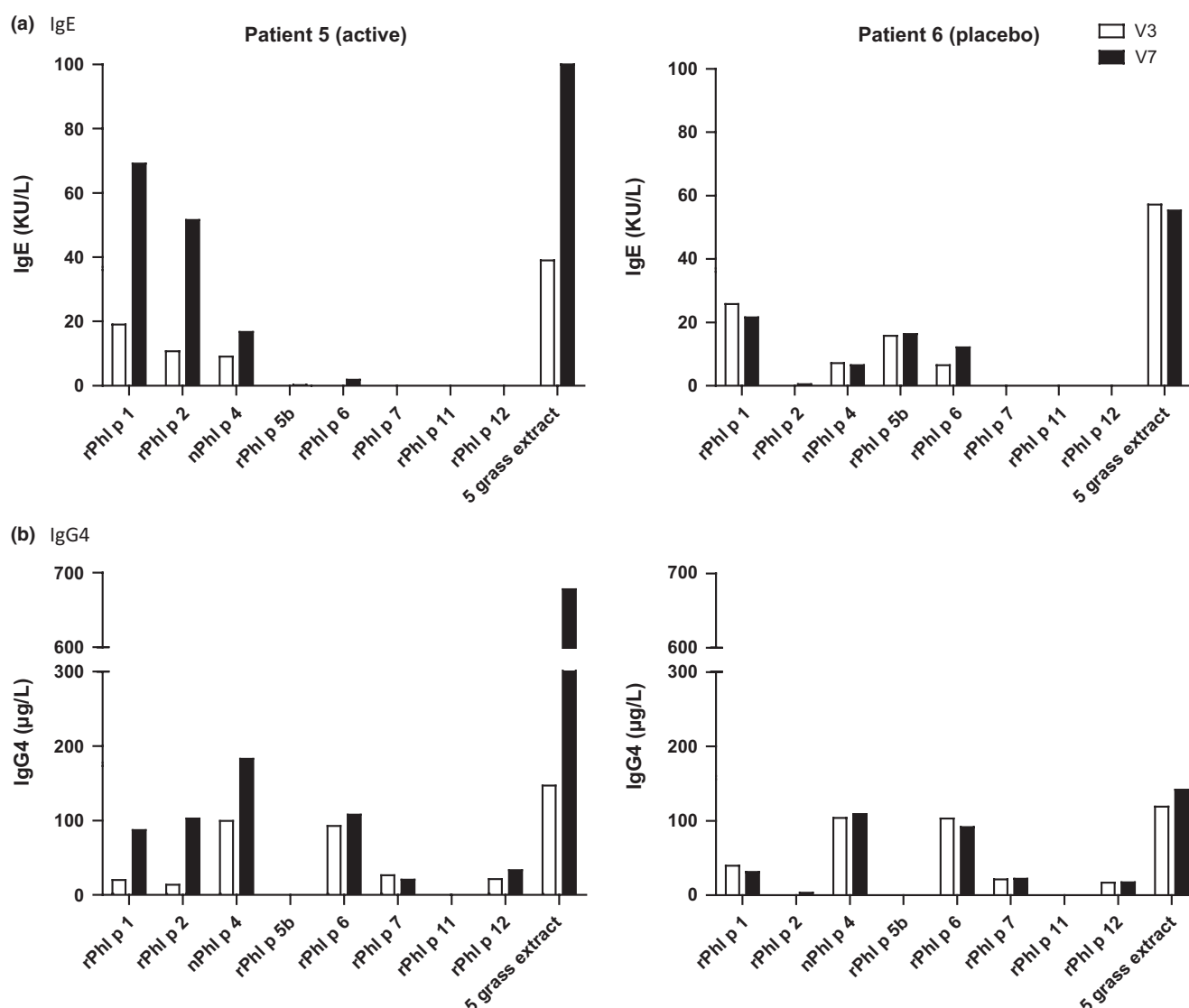


Fig. 3. Absence of IgE neosensitization to grass pollen allergens during sublingual immunotherapy. Seric IgEs (a) and IgG4s (b) specific for purified grass pollen allergens (as indicated) or a 5-grass-pollen extract were quantified by ImmunoCAP before (V3, white bars) and after (V7, black bars) treatment. Results are shown for two representative patients treated with either active (patient 5, left panels) or placebo (patient 6, right panels) tablets. NB: in a few patients from either one of the treatment groups, low levels (< 2.8 kU/L) of specific IgEs (e.g. directed to rPhl p 2 for patient 6 and to rPhl p 5b and rPhl p 6 for patient 5) became detectable after treatment likely as a consequence of allergen challenge in the chamber.

IgG4 or IgA titres after treatment in both nasal washes and saliva (Figs 4a and b, central and right panels) in patients from either group. Thus, those

local responses were not related to treatment, but rather to allergen (re)exposure during challenge in the chamber.

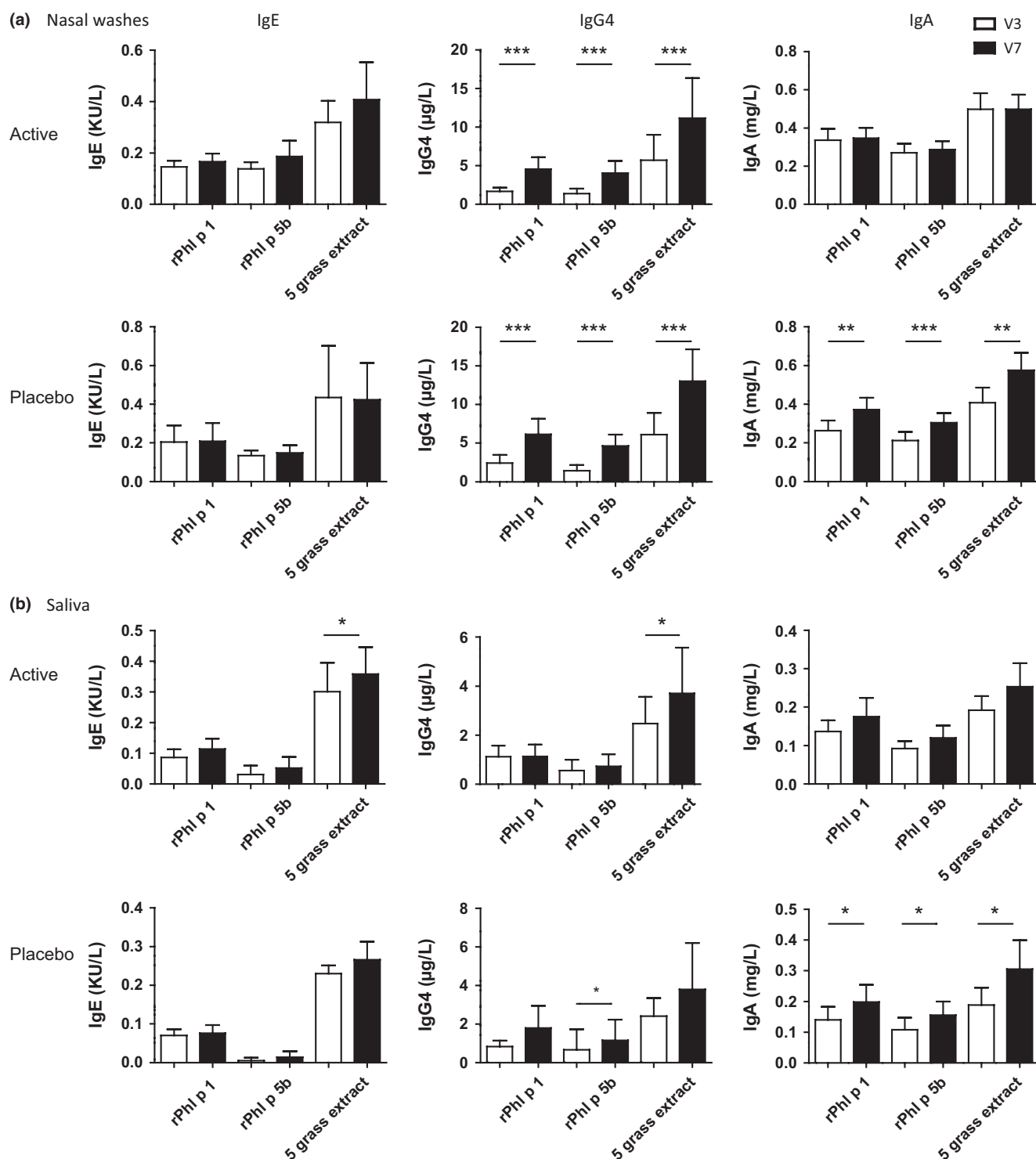


Fig. 4. Antibody responses in nasal washes and saliva during sublingual immunotherapy. IgEs, IgG4s and IgAs specific for rPhl p 1, rPhl p 5b and the 5-grass-pollen extract were quantified by ImmunoCAP in nasal washes (a) and saliva samples (b) collected from patients before (V3, white columns) and after (V7, black columns) treatment. Mean values $\pm$ SEM measured in patients receiving either active ($n = 42$) or placebo ($n = 40$) tablets are shown (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Wilcoxon matched-pairs signed rank test).

Changes in the functionality of specific seric antibodies during sublingual immunotherapy

The functionality of seric IgEs and IgG4s was investigated in terms of presence of IgE-blocking activity (Fig. 5a), CD23-dependent allergen-IgE binding to B lymphocytes (Fig. 5b) and affinity of IgEs, IgG3s and IgG4s to purified Dac g 1 or Dac g 5 allergens (Fig. 5c). In sera from patients belonging to the active group, the IgE-blocking activity increased by a mean 7.4-fold during treatment, while the corresponding increase in the

placebo group was only 1.8-fold ($P < 0.001$) (Fig. 5a). In contrast, allergen-IgE-FAB inhibition was unchanged by treatment in both groups (Fig. 5b). SPR analysis indicated no major changes in the affinity of seric IgG4s or IgEs for Dac g 1 (Fig. 5c, left panels) in either group, whereas a small but statistically significant increase was observed in the active group relative to placebo regarding the affinity of IgG4s or IgEs for Dac g 5 ($P < 0.01$ and $P < 0.05$, respectively, Fig. 5c, right panels).

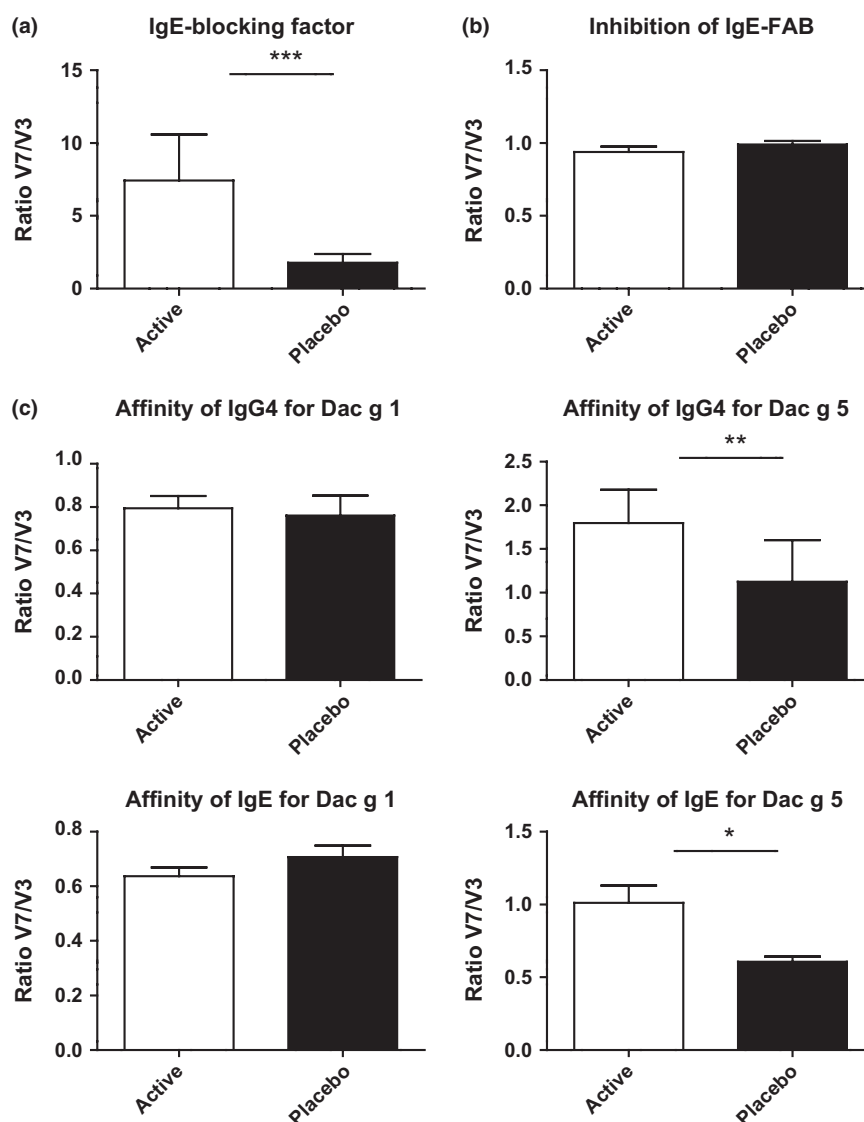


Fig. 5. Functional analyses of antibodies induced during sublingual immunotherapy. The functionality of antibodies was assessed by various methods in patients receiving either active (white columns, $n = 42$) or placebo (black columns, $n = 40$) tablets. (a) IgE-blocking factor was measured using anti-IgE-coated beads. (b) Inhibition of binding of IgE-allergen complexes to the low-affinity CD23 IgE receptor was assessed by the IgE-facilitated allergen binding assay. (c) Affinities of seric IgG4 and IgE antibodies for the major group 1 and group 5 allergens from *Dactylis glomerata* (i.e. Dac g 1 and Dac g 5, respectively) were measured by surface plasmon resonance. Mean $\pm$ SEM of ratios between post-treatment (V7) and pre-treatment (V3) values measured in patients receiving either active ($n = 42$) or placebo ($n = 40$) tablets is shown (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Mann-Whitney test).

Identification of a subgroup of immunoreactive patients clinically improved

The relationship between changes in antibody responses and clinical efficacy was assessed at an individual patient level. For each antibody analysis, we observed a clear-cut heterogeneity within active responder patients, with some of them exhibiting strong increases in titres, blocking activity and/or affinity of antibodies, whereas other clinical responders had antibody responses indistinguishable from the ones observed in the placebo group (Figs 6a–d). To further investigate this heterogeneity, a 20% subset of patients with the highest V7/V3 ratios was identified for each of the parameters tested in relationship to humoral responses. A total antibody score was calculated (Table 1), revealing that a subset of seven patients (representing 17% of treated patients) had strong antibody responses during SLIT detectable

in multiple analyses (i.e. with an antibody score $\geq 12/31$). Interestingly, these seven patients termed 'immunoreactive' (open circles in Figs 6a–d) exhibited a significant improvement in their ARTSS score (Fig. 6, Table 1). Nonetheless, other clinical responders among the active group do not exhibit such a clear induction of antibody responses during SLIT (Figs 6a–d, black dots on the upper side along the ordinate axis).

Discussion

Changes in IgE and IgG levels during allergen-specific subcutaneous or SLIT have been well documented [4, 6, 7] and proposed to be indicative of treatment efficacy. However, many open questions remain regarding (i) the impact of baseline IgE sensitization profiles on response to the treatment, (ii) the risk of *de novo* IgE sensitization after administration of extracts containing multiple

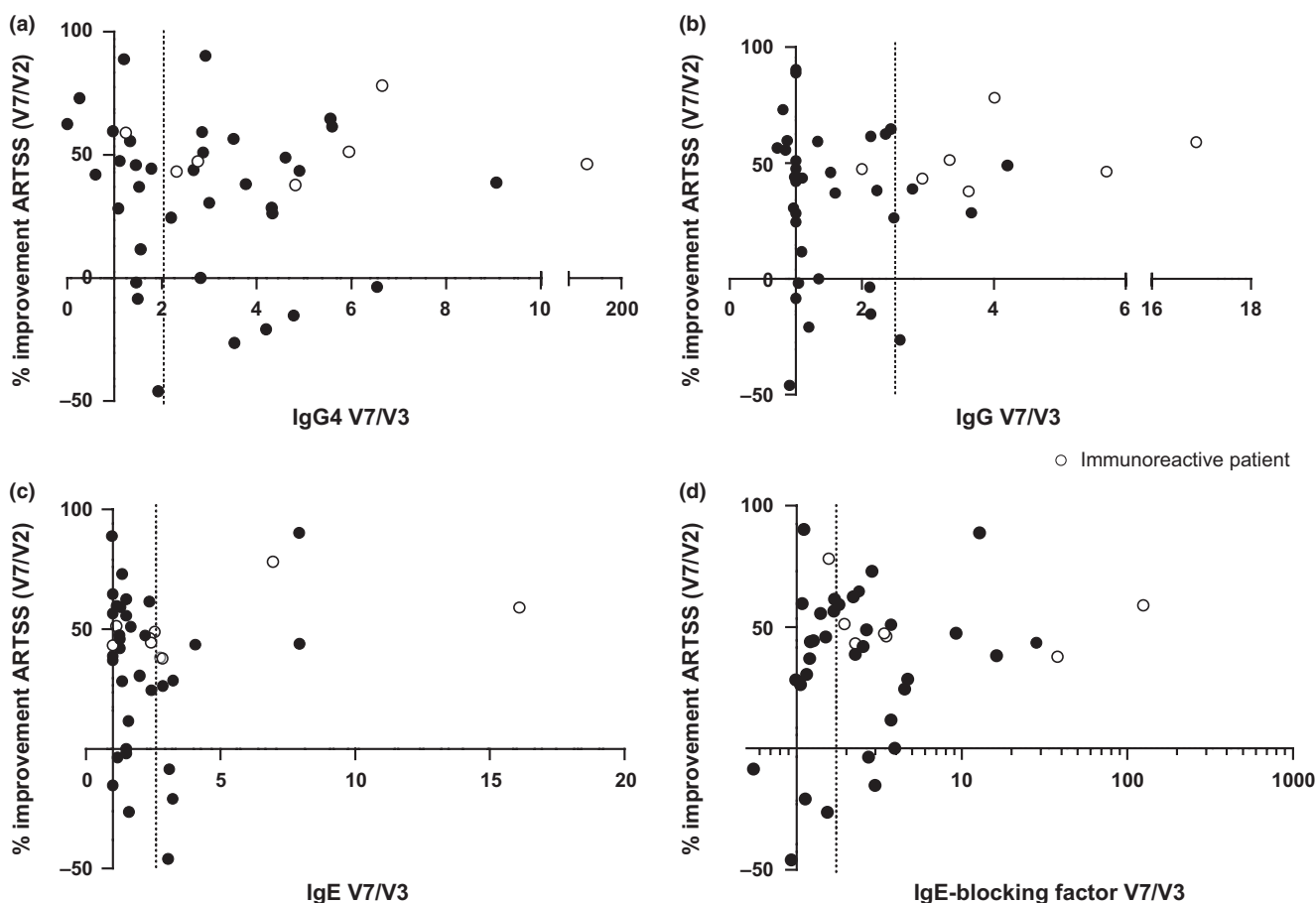


Fig. 6. Lack of correlation between clinical improvement and induction of antibody responses. IgG4s (a), total IgGs (b) and IgEs (c) reactive with the 5-grass-pollen extract were quantified in sera by ImmunoCAP before (V3) and after (V7) treatment. IgE-blocking factor (d) was measured using anti-IgE-coated beads. Clinical responses expressed for individual patients belonging to the active or placebo groups as percentages of average rhinoconjunctivitis total symptom score improvement after treatment were plotted against V7/V3 ratios measured for the indicated immune parameter. Vertical dotted lines represent the 90th percentile of V7/V3 ratios of the immune parameter measured in patients receiving placebo tablets. Open circles correspond to the seven immunoreactive patients from the active group exhibiting the highest V7/V3 ratios for various parameters related to humoral responses.

Table 1. Characteristics of immunoreactive clinical responders

Patient n°	IgG4 anti												IgG anti												IgG anti												Affinity				Clinical score % ARTSS improvement
	Phl p						5G						Phl p						5G						Phl p						5G						IgE- blocking factor	IgE			
																															Dac g 5	Dac g 1									
	1	2	4	5b	6	7	11	12	Extr	1	2	4	5b	6	7	11	12	Extr	1	2	4	5b	6	7	11	12	Extr	Dac g 5	Dac g 1												
7	1		1	1	1	1	1	1					1						1	1	1	1	1	1	1	1	1	1	1	16	46.3										
8										1		1	1	1			1	1	1	1	1	1	1	1	1	1	1		1	15	59.1										
9			1	1			1			1	1	1	1			1	1	1	1	1	1	1	1					1	1	14	47.4										
10		1		1	1			1			1			1	1	1	1		1			1	1			1				13	51.3										
11	1	1	1					1		1			1	1	1				1		1	1	1		1	1				13	37.8										
12		1	1	1	1		1												1	1	1	1	1				1			12	43.3										
13									1	1	1	1	1	1	1	1		1	1	1	1	1	1			1				12	78.2										

For each parameter considered, the 20% subset of patients with the highest V7/V3 ratios was scored as '1'. A total antibody score was subsequently calculated, confirming that seven patients included in the active group exhibit a strong upregulation of antibody responses with an impact on ≥ 12 parameters tested.

Herein, we addressed those questions using quantitative, qualitative and functional assays to monitor antibody responses induced during sublingual treatment with a 5-grass-pollen tablet. We took advantage of a cohort of 82 patients included in a double-blind, placebo-controlled study performed in a challenge chamber, thereby allowing a baseline evaluation of symptom scores and consequently, a precise determination of symptom improvement after treatment for each individual patient. Specifically, a comprehensive analysis of changes in IgAs, IgEs, IgGs and IgMs was performed in peripheral blood and mucosal fluids (saliva and nasal washes) using a panel of purified grass pollen allergens or a grass pollen extract, with an attempt to correlate such changes with clinical improvement and thus identify potential biomarkers of efficacy [27].

A wide spectrum of IgE sensitization profiles to pollen allergens was found among patients, in agreement with other studies [25, 32]. Importantly, clinical benefit was observed in patients irrespective of their pattern (i.e. narrow, intermediate or broad) of IgE sensitization. The latter observation does not support the hypothesis that patients with sensitization to a wide range of major and minor allergens may respond poorly to immunotherapy [33]. Interestingly, if anything, the best clinical responders in our study appear to be those exhibiting the broadest range of IgE sensitization, because the difference in percentages of ARTSS improvement in comparison with the placebo group was the most significant for patients exhibiting pattern III ($P = 0.007$, Mann–Whitney test). A 1.6–4.3-fold increase in pre-existing levels of allergen-specific seric IgEs was observed after 4 months of SLIT, without any induction of IgEs against allergens to which patients were unsensitized prior to treatment. This absence of *de novo* IgE sensitization observed in the present study supports and extends our previous observation made in a large cohort of mite allergic patients receiving SLIT [26]. Also, we did not detect any significant upregulation of specific IgEs in mucosal tissues during successful SLIT, documenting why the well-known boost observed in existing seric IgE titres during immunotherapy is well tolerated by the patients. Those data can be explained by the tolerogenic bias of the oral immune system [4, 34] and confirm that the sublingual route is safer when compared with subcutaneous administration [23, 25, 26].

Serum allergen-specific IgG4 titres increased during treatment, most particularly against group 1 and group 5 allergens, in agreement with other studies [3, 13, 16, 17, 35]. Importantly, a clear heterogeneity between patients was evidenced, with the identification of a sub-

set comprising 17% of all patients exhibiting strong antibody responses during SLIT. Whereas such immunoreactive patients were clinically improved, other clinical responders had no detectable antibody responses distinguishable from placebo-recipients, suggesting that other mechanisms beyond blocking antibodies can contribute to SLIT efficacy. Evidence for such a subset of immunoreactive patients with a strong humoral response following SLIT was also reported in our recent study conducted in a large cohort of house dust mite patients [26]. Collectively, these results suggest that the induction of specific IgG4s may benefit some patients, but cannot be used as a biomarker of SLIT efficacy applicable to each individual patient.

No significant increase in specific IgA1, IgA2, IgG1, IgG2, IgG3 and IgM antibodies was detected in post-treatment serum samples, although induction of IgG1 and IgA responses in the course of subcutaneous immunotherapy or SLIT has been reported by others [20, 35–40]. These differences could possibly be explained by the longer duration of immunotherapy in those latter studies relative to the present one (i.e. 1–2 years of treatment vs. 4 months). We also investigated the functionality of IgGs, recently proposed as being relevant to allergen immunotherapy (AIT) efficacy, including their affinity to allergens or potential blocking activities [19]. Noteworthy, as SPR analyses were carried out on purified polyclonal antibodies, our results rather reflect changes in avidity more than affinity of antibodies. In the present study, only the IgE-blocking factor significantly increased post-treatment, with no or minor concomitant change in IgE-FAB or antibody avidity observed. This latter result is in agreement with a

previous study concluding that AIT has no effect on the affinity of allergen-specific IgE, IgG1 or IgG4 antibodies [41]. It is noteworthy, however, that the observed increase in IgE-blocking factor did not correlate with improvement in symptom scores.

In conclusion, our results confirm that SLIT is efficacious in patients exhibiting different IgE sensitization profiles, in particular in those sensitized to both major and minor allergens. Clinical responders include both patients with strong allergen-specific seric antibody responses, but also patients lacking such responses. The latter observation suggests that additional immune mechanisms distinct from blocking antibodies are involved in SLIT efficacy, making it difficult to rely upon humoral responses to predict clinical responses at an individual patient level.

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Conflict of interest

Véronique Baron-Bodo, Stéphane Horiot, Aurélie Lautrette, Henri Chabre, Sylvie Galvain, Robert Kenneth Zeldin and Philippe Moingeon are employees at Stallergenes.

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