

Immunological comparison of allergen immunotherapy tablet treatment and subcutaneous immunotherapy against grass allergy

K. Aasbjerg¹, V. Backer¹, G. Lund², J. Holm², N. C. Nielsen², M. Holse², V. R. Wagtman² and P. A. Würtzen²

¹Respiratory Research Unit, Department of Lung Medicine, Copenhagen University Hospital Bispebjerg, Copenhagen, Denmark and ²ALK, Hørsholm, Denmark

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Summary

Background IgE-mediated allergic rhinitis to grass pollen can successfully be treated with either allergen immunotherapy tablets (SLIT tablet) or SQ-standardized subcutaneous immunotherapy (SCIT). The efficacy of these two treatment modalities for grass allergy is comparable, but the immunological mechanisms may differ. ClinicalTrials.gov ID: NCT01889875.

Objectives To compare the immunological changes induced by SQ-standardized SCIT and SLIT tablet.

Methods We randomized 40 individuals with grass pollen rhinitis into groups receiving SCIT, SLIT tablet, or neither and followed them for 15 months with regular serum measurements of specific IgE, IgG4, IgE-blocking factor, facilitated antigen presentation (FAP), and basophil activation test (BAT). Nasal challenges were used to assess changes in nasal sensitivity.

Results After 15 months of treatment IgG4, IgE-blocking factor, FAP, and BAT values differed significantly in both SCIT and SLIT-tablet treatment groups when compared to the control group. Both SCIT and SLIT-tablet groups were significantly different from the control group after 1–3 months of treatment. In general, the changes induced by SCIT reached twice that of SLIT tablet, with the exception of specific IgE where SLIT tablet induced initial threefold increase compared with SCIT. A slight but significant increase in IgE and BAT after season was seen only in the control group. Significant differences between SCIT and SLIT tablet were observed early, but the differences diminished with the length of treatment, especially for FAP inhibition.

Conclusions Both SCIT and SLIT tablet induce significant changes in specific antibodies (IgE and IgG4) and competition assays (IgE-blocking factor, FAP, and BAT). Overall, SCIT induced larger (two- to threefold) changes than SLIT tablet, with the exception of FAP, where SLIT tablet showed a gradual increase ending at the same level as SCIT. Maximal change was generally reached after 3 months' treatment.

Keywords basophil activation, basophil sensitivity, blocking factor, facilitated antigen presentation, IgE, IgG4, immunotherapy, nasal challenge, *Phlerum pratense*, rhinitis, SCIT, SLIT tablet

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Correspondence:

Kristian Aasbjerg, Respiratory Research Unit, Department of Lung Medicine, Copenhagen University Hospital Bispebjerg, Entrance 66, Copenhagen NV DK-2400, Denmark. E-mail: kristian@aasbjerg.dk

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Introduction

World-wide, more than 400 million people have allergic rhinitis, and sick leave due to this condition costs societies billions of dollars annually [1]. Allergic rhinitis is most commonly treated with oral antihistamines and intranasal corticosteroids [2]. For moderate-to-severe rhinitis with poor control on topical treatment,

immunotherapy should be considered [3]. Allergen immunotherapy is the only disease-modifying treatment available for IgE-mediated allergy.

Immunotherapy is most commonly administered with subcutaneous injections of SQ-standardized allergen extracts [subcutaneous immunotherapy (SCIT)] and, to a lesser extent, as sublingual drops (SLIT). However, other options now exist with the newly developed sublingual

allergen immunotherapy tablets (SLIT tablet). The clinical effect of SLIT tablet [4] and SCIT [5] against grass pollen allergy has been demonstrated in large double-blind placebo-controlled registration trials. These studies report significant reductions in symptom scores (SLIT tablet 30% and SCIT 32%) and seasonal rescue medication scores (SLIT tablet 38% and SCIT 41%) when compared to placebo [4, 5], persisting several years after withdrawal [4, 6]. Immunotherapy may prevent new sensitizations and progression of rhinitis to asthma [7–11]. Allergen immunotherapy is generally well tolerated with few side-effects, although a potential risk of anaphylactic reactions exists [12]. A review of recent parallel meta-analyses on immunotherapy for grass pollen rhinitis suggests that the safety profile of SLIT tablet is superior to that of SCIT [13].

The recommended length of immunotherapy with both SCIT and SLIT tablet is 3–5 years [2], although the route of administration and the doses of allergen differ substantially. SCIT involves an up-dosing phase lasting 12–15 weeks with one or more weekly injections of increasing doses of allergen followed by a maintenance dose every 6–10 weeks. SLIT tablet starts with the daily maintenance dose, resulting in high cumulative doses. It is largely unknown whether the immunological mechanisms involved in the clinical effect are the same.

The changes in immunology have been extensively investigated for SCIT, and the most pronounced patterns are (1) the induction of blocking factor, (2) a shift in Th1/Th2 balance towards Th1, and (3) induction of regulatory T cells. Similar mechanisms seem to be in play for SLIT tablet [14–16]. Increases in allergen-specific non-IgE antibodies have been demonstrated in large trials of both SCIT and SLIT-tablet treatment [17, 18], and studies have demonstrated that serum antibodies can reduce *in vitro* reactions mimicking allergic responses, such as IgE binding to allergen, IgE-facilitated antigen presentation, and basophil activation [19–22], suggesting that both humoral and cellular effects play a role [23].

The aim of the present study was to compare the effect of SLIT tablet and SCIT on antibody titres (IgE and IgG4), on allergen-IgE interaction in competition assays (IgE-blocking factor and FAP inhibition) and on effector cell activation (BAT). Nasal challenges were included to assess the changes in nasal response to grass pollen allergen.

Materials and methods

We randomized 40 patients to 15 + 15 + 10 in SCIT, SLIT tablet, and control, respectively, and followed them for 15 months' treatment. The clinician performed the evaluation on inclusion, the nasal challenge, and the non-blinded treatment, whereas all *in vitro* tests were performed on blinded samples.

In all participants, serum for antibody testing was obtained at –3, 0, 1, 2, 3, 6, 7, 10, 12, and 15 months and basophils at –2, 1, 3, 6, 7, 10, 12, and 15 months. Nasal challenges were performed at –2, 3, 10, and 15 months. For all laboratory analysis, pre-treatment values were subtracted from all post-treatment values (Δ values) before analyses.

Inclusion criteria were (1) history of rhinitis with hayfever symptoms during the grass pollen season, (2) a skin-prick-test-verified grass pollen allergy (*Phleum pratense*), and (3) eligibility for immunotherapy based on Allergic Rhinitis and its Impact on Asthma (workgroup; ARIA) guidelines [2].

Exclusion criteria were (1) current long-term systemic steroid treatment, (2) previous immunotherapy, (3) asthma and outside-season airway hyperresponsiveness (AHR) (September 2011), (4) pregnancy, (5) negative RAST for grass, and (6) treatment side-effect.

In addition, participants who did not complete the SCIT treatment and SLIT-tablet-treated participants with an adherence rate less than 75% were excluded from the final data analysis.

We screened 78 individuals: 13 (17%) were diagnosed with AHR, 25 were excluded due to various other reasons, and the remaining 40 were randomized. After enrolment, 4 additional patients were excluded due to lack of adherence; of these, 2 had developed clinically significant side-effects to SCIT. A CONSORT diagram is included in the Appendix S1.

Specific IgE was measured both at baseline and at follow-up using methods described previously [11, 17, 19].

IgG4 measurements were taken on a Phadia CAP instrument according to the supplier's protocol for analysis of grass-specific IgG4 in human serum (Phadia AB, Uppsala, Sweden). The method was validated on sera being pre-diluted (1 : 50) in diluent.

IgE-blocking factor

The presence of components blocking IgE-allergen binding (i.e. IgE-blocking factor) was measured as previously described [11, 19]. Briefly, the assay is a 2-step competition assay, based on 2 specific IgE determinations from the same serum sample. Step 1 determines the total amount of specific IgE capable of binding to allergen when all competing components have been rinsed off (washed away after the first assay step/incubation). This corresponds to a standard specific IgE quantification. Step 2 determines the amount of specific IgE bound to allergen in the (possible) presence of competing components, including antibodies of unspecific isotypes. The results of the assay are reported as 1–(Step 2/Step1), which is named IgE-blocking factor. The IgE-blocking factor is a dimensionless number which theoretically

varies from 0 (no presence of any IgE-blocking factors) to 1 (all IgE blocked from binding to allergen).

Facilitated antigen presentation inhibition assay

The procedure has been published previously [19, 24]. In short, a stock indicator serum pool from 3 grass pollen allergic donors, containing high concentrations of allergen-specific IgE (> 100 KU/mL, measured by the Advia Centaur method, ALK, Hoersholm, Denmark), was pre-incubated with *P. pratense* allergen extract (0, 0.3, and 0.6 µg/mL) at 37°C for 1 h alone (volume allergen : indicator : buffer = 1 : 1 : 1) or in the presence of participant sera (volume allergen : indicator : participant serum = 1 : 1 : 1). Next, EBV-transformed B cells were added at 10⁵ cells per sample (volume allergen : indicator : participant serum : B cells = 1 : 1 : 1 : 1) and were further incubated for 1 h at 4°C before washing and detection of surface binding of allergen-IgE complexes by flow cytometry using a FITC-labelled anti-IgE polyclonal antibody (STAR147F; Serotec, Dusseldorf, Germany). All participant samples were analysed in duplicate.

Basophil activation test

Basophils in full blood were stimulated with *P. pratense* allergen extract (0.00015–1500 ng/mL), and the expression of activation markers was measured by flow cytometry with a modification of a previously published method [23, 25]. Basophils were identified as CCR3 and CD203c double-positive cells with exclusion of cells with high side scatter (SSC). The fraction of allergen-induced double-positive cells with high expression of CD63 and CD203c was compared in terms of percentage of activation. Based on dose–response curves for each donor, allergen sensitivity was determined by calculating EC50 (allergen concentration giving a 50% of maximal activation, ng/mL). Values of EC50 were inverted (basophil sensitivity) before analyses according to Nopp et al. [26]. Detailed descriptions of gating strategies and activation markers are included in the Appendix S1.

Nasal challenge

On arrival at the hospital, participants were asked to rinse their nostrils using a Sinus-Rinse Kit (Neilmed Pharmaceuticals, Santa Rosa, CA, USA); 30 min after, the nasal challenge was performed using an aqueous solution of Aquagen 225 (ALK) grass pollen extract (*P. pratense*) with a fixed dose of 5000 SQ-U delivered to each nostril via an Aptar (Aptar Pharma, Radolfzell, Germany) Bi-Dose system [27]. Patients were allowed to wipe their noses but were asked not to blow them. Symptom scores were obtained 30 min post-challenge using a visual analogue

scale (VAS) with questions on congestion, runny nose, nose itch, palate itch, eye itch, and total symptoms.

Randomization

A list of 40 allocation numbers was created. The list was then randomized using <http://www.random.org/lists/>, and each allocation number was printed in the randomized order on a separate sheet of paper that was then placed in sealed envelopes. The envelopes were then given to a nurse not participating in the study, who was instructed to hand an envelope to each participant without looking inside the envelope or changing the order of envelopes.

Skin prick test

Skin prick test (SPT) was carried out using standard ALK SoluPrick (ALK) allergen kits consisting of *Betula verrucosa*, *Phleum pratense*, *Artemisia vulgaris*, Horse, Dog, Cat, *Dermatophagoides pteronyssinus* and *farinae*, *Claudosporidium herbarium*, and *Alternaria alternata* (ALK). Weals more than 3 mm in diameter were considered positive.

Airway hyperresponsiveness

Mannitol challenge test was performed using inhaled capsules of mannitol in increasing doses aiming at a cumulative dose of 635 mg. FEV₁ was measured after each inhalation. The test was positive if FEV₁ dropped 15% or more [28, 29].

SCIT

Treatment performed using ALK Alutard 225 '*P. pratense*' (ALK) start-up kit with four ampoules each containing 5 mL solution of increasing concentrations of grass pollen extract. The first injection consisted of 20 SQ-U and gradual up-dosing every week for 15 weeks up to a maintenance dose of 100 000 SQ-U given every 2 months throughout the study. All injections were given within ± 2 weeks of the optimal schedule, in accordance with the guidelines.

SCIT adherence

All individuals in the SCIT group received injections according to the defined schedule ± 1 week as per guidelines.

SLIT tablet

Treatment was with ALK Grazax 75 000 SQ-T sublingual tablets. Patients placed one tablet under the tongue

throughout the study. All patients were asked to present their tablet packages at every hospital appointment. The tablets were then counted, and patients received the number of tablets required for the subsequent therapy period. An estimated adherence rate was calculated for each patient as the number of tablets actually taken divided by the number expected to be taken.

SLIT-tablet adherence

The number of tablets needed for each participant was approximately 450; the average number used was 400, and the mean compliance for the SLIT-tablet-treated group was estimated as 89.2% (95% CI: 82.5–95.8).

Statistics

Statistical analyses were carried out using SAS Statistical Software Package, version 9.3 (SAS Institute Inc., Cary, NC, USA) for Windows. P -values < 0.05 were considered significant. Mixed regression models were used to analyse the development of measures over time, with visit, treatment group, and the interaction of both as covariates. The area under the curve (AUC) was estimated for each biomarker as a continuum over time; using trapezoid methods on the delta values, the AUC was calculated for each individual who completed the entire study and compared using analysis of variance. At the final visit, Spearman correlations between fold increases in each assay were calculated for each treat-

ment arm, and linear regression models with backwards elimination were used to assess whether fold increase in IgG4 and treatment influenced fold increase in IgE-blocking factor and facilitated antigen presentation and basophil sensitivity.

Ethics

The study was approved by the Danish Board of Ethics, approval no. H-3-2010-042, and all subjects signed informed consents before any study procedure.

Results

Specific IgE to *Phleum pratense*

Specific IgE in the control group increased significantly compared with baseline during the pollen season ($P < 0.05$), and levels did not return to baseline until 6 months later (Fig. 1). *P. pratense*-specific IgE in the SCIT group increased significantly compared with the control group 1–2 months after treatment start ($P < 0.05$) and dropped gradually towards the end of the study ($P > 0.05$). In the SLIT-tablet group, IgE increased significantly compared with the control group immediately after treatment start ($P < 0.05$), followed by a gradual decline towards the end of the study. At 15 months, IgE in the SLIT-tablet group was no longer significantly different from controls. The initial increase in the SLIT-tablet group was quicker (1 month

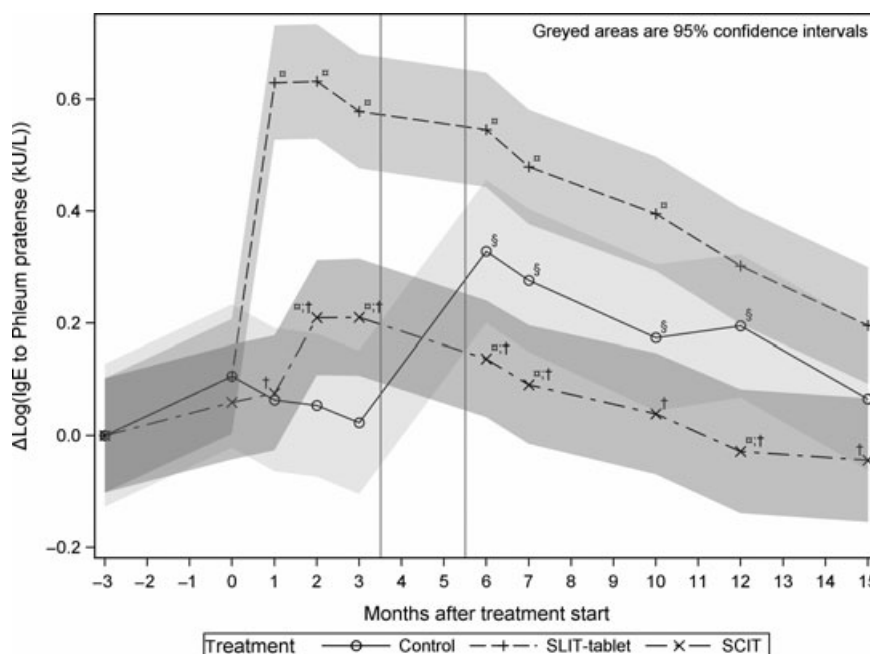


Fig. 1. Change in serum IgE to *Phleum pratense* compared with baseline. □, Significantly different from control group. †, Significantly different when compared to SLIT tablet. Vertical lines indicate the peak grass (*Phleum pratense*) pollen season (May/June) in 2011.

compared with 1–2 months) and threefold higher compared with that in the SCIT group ($P < 0.05$). The area under the curve (AUC) was 2.6, 0.7, and 7.0 for the control, SCIT, and SLIT-tablet groups, respectively, with SCIT vs. controls $P = 0.15$, SLIT tablet vs. controls $P < 0.01$ and SCIT vs. SLIT tablet $P < 0.01$.

IgG4 antibodies

The kinetics for *P. pratense*-specific IgG4 and IgE-blocking factor followed parallel paths, and a statistically significant difference from the control group was observed at similar time points (see IgE-blocking factor and Fig. 2). The maximum level of IgG4 was reached towards the end of the study for both active groups, with values of 0.60 in the SLIT-tablet group, compared with 1.71 in the SCIT group ($P < 0.05$). The AUC was 0.3, 19.5, and 7.1 for the control, SCIT, and SLIT-tablet groups, respectively, with SCIT vs. controls $P < 0.01$, SLIT tablet vs. controls $P < 0.01$, and SCIT vs. SLIT tablet $P < 0.01$.

IgE-blocking factor

In the control group, values for IgE-blocking factor did not change significantly during the entire study (Fig. 3), although a slight, non-significant increase was seen after the pollen season. The SCIT group reached a maximum level of around 0.50 during the first 3 months of treatment, the change being significantly different from

the control group after 2 months of treatment ($P < 0.05$). The SLIT-tablet group increased gradually and was significantly higher than the control group 3 months after treatment start ($P < 0.05$) with an IgE-blocking factor value of 0.18 and continued to increase throughout the study, reaching 0.27 by the end of the study. The initial increases for SCIT and SLIT tablet were not significantly different ($P > 0.05$) during the first 2 months of treatment, but after 3 months, the IgE-blocking factor for SCIT was significantly higher than for SLIT tablet ($P < 0.05$). Towards the end of the study, IgE-blocking factor for SLIT tablet was about half that of SCIT. The AUC for the control, SCIT, and SLIT-tablet groups was 0.7, 6.4, and 2.9, respectively, with SCIT vs. controls $P < 0.01$, SLIT tablet vs. controls $P = 0.03$ and SCIT vs. SLIT tablet $P < 0.01$.

Facilitated antigen presentation

In the control group, no inhibition for IgE-facilitated antigen presentation was seen during the entire study (Fig. 4). The SCIT group reached maximum inhibition 3 months after treatment start at 22.5% inhibition ($P < 0.05$), and no significant change was observed for the rest of the study. The SLIT-tablet group increased significantly compared with the control group after 3 months of treatment ($P < 0.05$) and continued to rise for the remainder of the study, reaching 17.2% inhibition after 15 months of treatment ($P < 0.05$). SLIT-tablet FAP inhibition was significantly lower than SCIT in

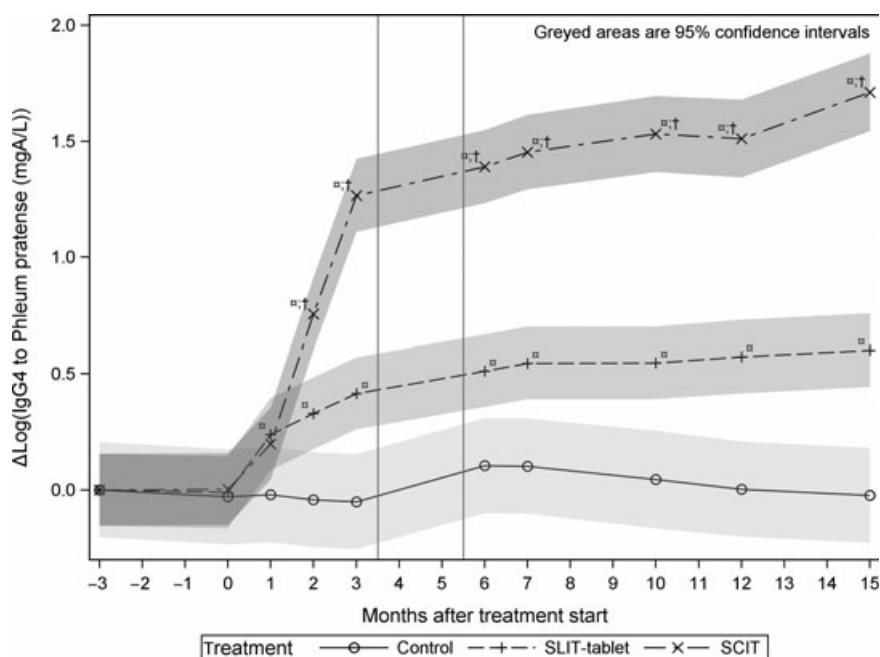


Fig. 2. Change in serum IgG4 to *Phleum pratense* compared with baseline. □, Significantly different from control group. †, Significantly different when compared to SLIT tablet. Vertical lines indicate the peak grass (*Phleum pratense*) pollen season (May/June) in 2011.

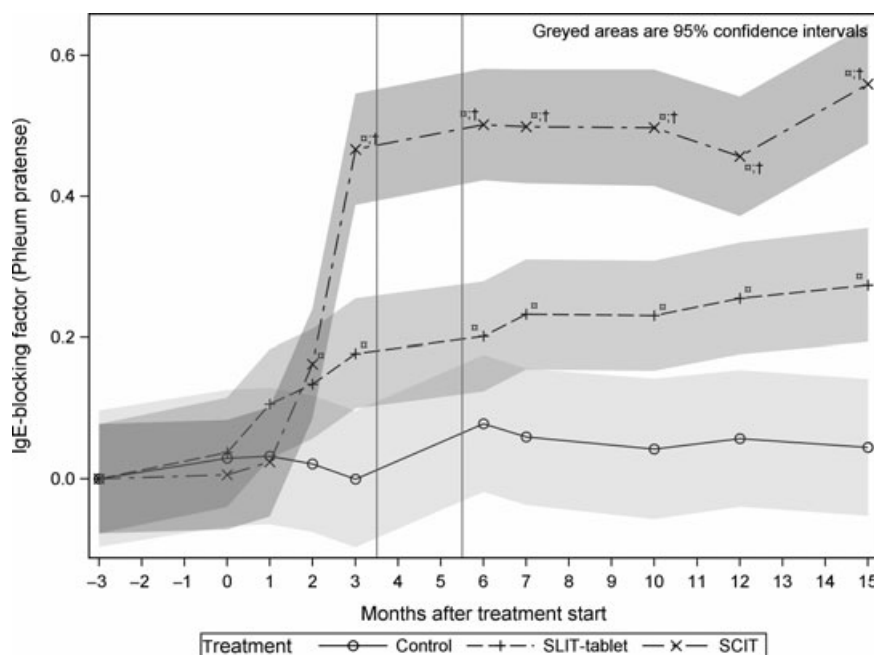


Fig. 3. Change in serum IgE-blocking factor to *Phleum pratense*. □, Significantly different from control group. †, Significantly different when compared to SLIT tablet. Vertical lines indicate the peak grass (*Phleum pratense*) pollen season (May/June) in 2011.

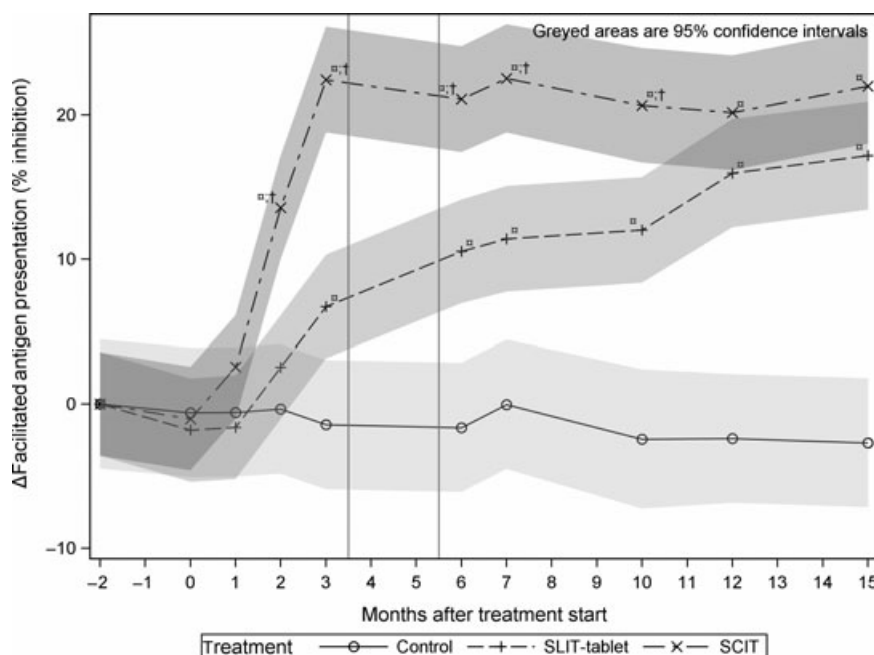


Fig. 4. Change in % inhibition of facilitated antigen presentation. Vertical lines indicate the peak grass (*Phleum pratense*) pollen season (May/June) in 2011.

the period 3–10 months of treatment, but not from 12 months and throughout the rest of the study. The AUC for the control, SCIT, and SLIT-tablet groups was –22.2, 298.1, and 155.9, respectively, with SCIT vs. controls $P < 0.01$, SLIT tablet vs. controls $P < 0.01$, and SCIT vs. SLIT tablet $P < 0.01$.

Basophil sensitivity (EC_{50})

In the control group, basophil sensitivity increased significantly from baseline to after the pollen season ($P < 0.05$), but quickly returned to baseline values (Fig. 5). The sensitivity in the SCIT group was

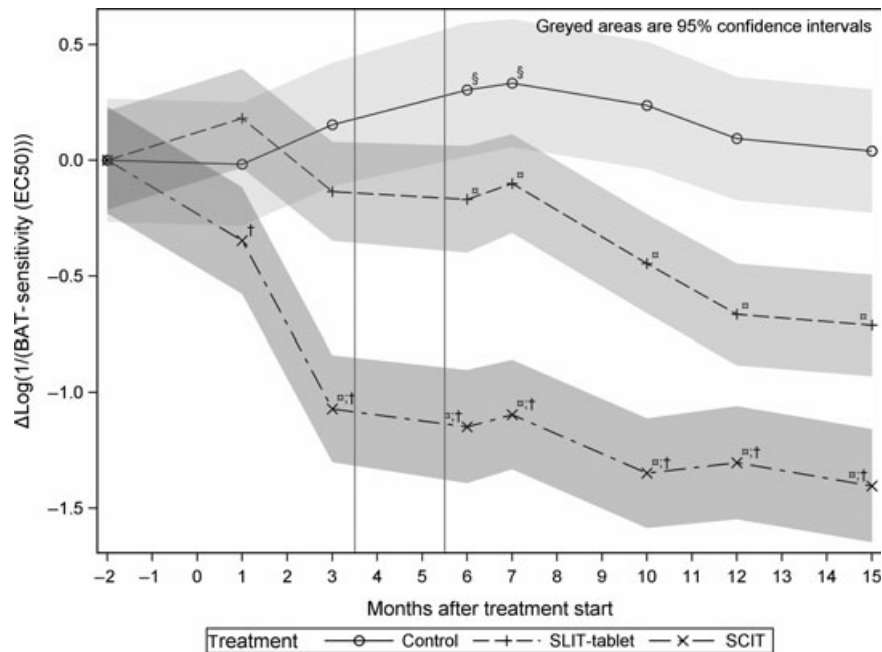


Fig. 5. Change in basophil activation test (BAT; EC50), compared with baseline. §, Control group significantly different from baseline (treatment months = 0). □, Significantly different from control group. †, Significantly different when compared to SLIT tablet. Vertical lines indicate the peak grass (*Phleum pratense*) pollen season (May/June) in 2011.

significantly lower than in the controls after 1 month of treatment ($P < 0.05$). The largest drop to -1.07 was seen after 1–3 months of treatment. The sensitivity reached -1.4 at the end of the study. The sensitivity in the SLIT-tablet group was significantly lower than in the control group immediately after the pollen season ($P < 0.05$) and dropped markedly after 7 months of treatment reaching -0.71 at the end of the study. SCIT was significantly lower after 1 month of treatment when compared to SLIT tablet, and although the differences diminished towards the end of the study, they remained significant. The AUC for the control, SCIT, and SLIT-tablet groups was 2.4, -17.2 , and -4.1 , respectively, with SCIT vs. controls $P < 0.01$, and SLIT tablet vs. controls $P = 0.01$, and SCIT vs. SLIT tablet $P < 0.01$.

Nasal response to *Phleum pratense* allergen extract

Symptom scores dropped to -55.1 (95% CI: -77.8 to 32.3) in the SCIT group after 3 months of treatment ($P < 0.05$) and to -6.2 (95% CI: -28.5 to 15.7) in the SLIT-tablet group, although this was not significant. After 15 months, the value for SCIT was -56.8 (95% CI: -81.7 to -31.9 ; $P < 0.05$) and -32.6 (95% CI: -55.1 to 10.1 ; $P > 0.05$) for SLIT tablet. The AUC for the control, SCIT, and SLIT-tablet groups was 42.8, -777.3 , and -360.5 , respectively, with SCIT vs. controls $P < 0.01$, SLIT tablet vs. controls $P = 0.16$, and SCIT vs. SLIT tablet $P = 0.13$. (Fig. 6)

Correlations between assays in the final visit

In the SLIT-tablet group, significant correlations were found between fold increase in IgE-blocking factor and IgG4 ($P = 0.01$; $R = 0.69$), as well as FAP and IgG4 ($P = 0.02$; $R = 0.66$). In the SCIT group, only IgG4 and IgE-blocking factor correlated significantly ($P < 0.05$; $R = 0.64$). In the IgE-blocking factor and FAP assays, only IgG4 influenced the readout ($P < 0.01$), whereas treatment did not. In the BAT assay, only treatment influenced the readout ($P < 0.01$), whereas IgG4 did not.

Baseline values of IgE, IgG4, IgE-blocking factor, facilitated antigen presentation, and basophil activation (EC50) were tested for significant differences between groups at baseline, and no difference could be observed ($P > 0.05$; see Table 1).

Discussion

We followed 40 participants with seasonal allergic rhinitis to grass pollen over 15 months to compare the immunological effect of SLIT tablets and SCIT. After enrolment, 15 were treated with SCIT and 15 with SLIT tablets and 10 were followed as a control group. Serum-specific IgE, IgG4, IgE-blocking factor, FAP, and basophil sensitivity determined by basophil activation (BAT) were measured regularly throughout the study. Most of the immunological changes induced by SCIT and SLIT tablets were significantly different from the untreated controls, and changes following SCIT treatment were more

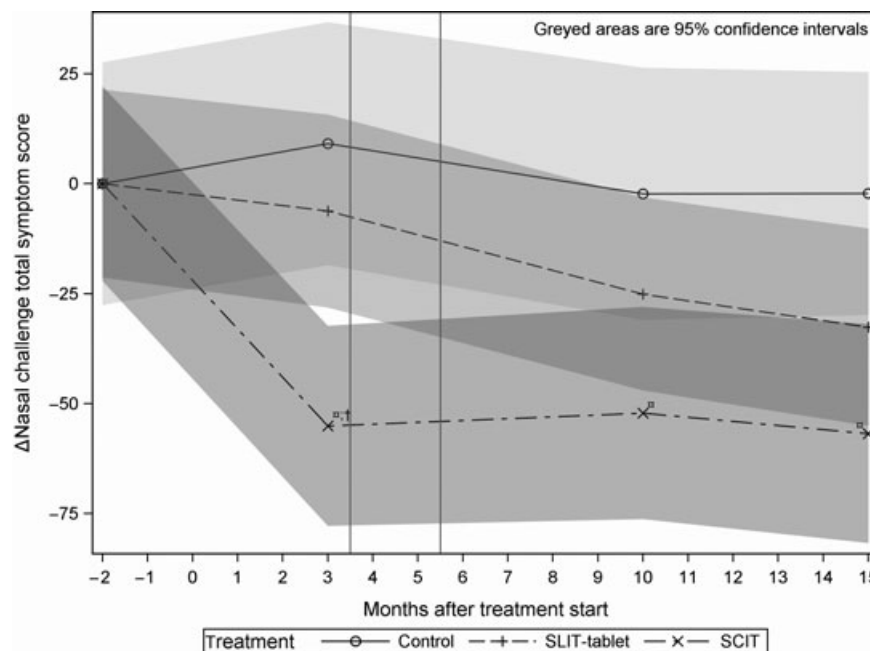


Fig. 6. Development in total symptoms visual analogue scale (VAS) post-nasal challenge. §, Control group significantly different from baseline (treatment months = 0). □, Significantly different from control group. †, Significantly different when compared with SLIT tablet. Vertical lines indicate the peak grass (*Phleum pratense*) pollen season (May/June) in 2011.

Table 1. Overview of population *Phleum pratense*-specific IgE, IgG4, IgE-blocking factor, basophil sensitivity, facilitated antigen presentation, and skin prick test parameters at baseline

	Control	SCIT	SLIT tablet	All	P value
Total number of participant(s)	9	14	15	38	
Number of female(s)	4 (44.4%)	3 (21.4%)	1 (6.7%)	8 (21.1%)	> 0.05
Mean age of all participant(s)	36.4 (20–63) year(s)	34.6 (20–59) year(s)	31.5 (22–46) year(s)	33.8 (20–63) year(s)	> 0.05
IgE (kU/L)	21.4 (5.5–37.4)	40.9 (13.0–68.8)	31.4 (19.1–43.6)	28.2 (10.2–46.2)	> 0.05
Skin prick test (diameter, mm)	9.3 (7.9–10.8)	9.2 (8.4–10.0)	9.3 (8.8–9.9)	9.4 (8.4–10.5)	> 0.05
IgG4 (mgA/L)	0.2 (0.1–0.3)	0.6 (0.2–1.0)	0.5 (0.2–0.7)	0.5 (0.3–0.6)	> 0.05
IgE-blocking antibodies	−0.2 (−0.3 to 0.1)	−0.1 (−0.2 to 0.0)	−0.2 (−0.3 to 0.0)	−0.2 (−0.2 to 0.1)	> 0.05
Basophil sensitivity (EC50)	0.5 (0.0–1.0)	0.4 (0.1–0.7)	0.7 (0.3–1.1)	0.5 (0.3–0.7)	> 0.05
Facilitated antigen presentation	39.2 (29.0–49.4)	35.6 (31.3–39.8)	43.1 (36.3–49.9)	39.3 (35.6–42.9)	> 0.05
Nasal challenge	80.7 (52.5–108.8)	90.9 (70.6–111.1)	71.8 (45.1–98.5)	80.9 (67.5–94.3)	> 0.05

SCIT, subcutaneous immunotherapy; SLIT tablet, allergen immunotherapy tablets.

pronounced than for SLIT-tablet treatment. The difference between SCIT and SLIT tablets seemed to diminish with the length of treatment (especially for FAP), suggesting that the changes regress towards the same level over time. Moreover, the pollen season significantly changed baseline levels of specific IgE and basophil sensitivity in the control group, indicating that the seasonal pollen exposure may generally affect immunological assays so that high pollen counts increase specific IgE as well as the sensitivity of basophils.

Specific IgE

Several studies have confirmed that immunotherapy induces increased specific IgE levels in serum [14, 21,

30]. Blunting of the seasonal increase in IgE has been demonstrated for various grass allergens [20, 31, 32] and for different allergen extracts in SCIT [33, 34]; our data seem to confirm these findings. Similarly, a continuous reduction in IgE levels, after the early increase was observed following SLIT-tablet treatment, suggests that the seasonal increase observed in the control group is overruled by both treatments (Fig. 1), in line with previous reports [31]. The difference in specific IgE between the AIT group and the SCIT and control groups was not significant (Table 1; $P > 0.05$). Furthermore, these numeric differences diminish substantially when considering delta values as numeric, inter-personal differences practically disappear in the relative changes.

IgG4 and IgE-blocking factor

In serum from the control group, we found no significant change in IgG4 (Fig. 2) or IgE-blocking factor (Fig. 3) compared with baseline. However, in the SIT groups, we found a significant increase in both assays during the first 3 months of treatment, confirming the findings of several other studies [20, 30, 31, 35, 36].

For SLIT-tablet treatment, IgG4 antibodies increased gradually and reached a $\Delta\log_{10}$ value of about 0.5 by the end of the study. This is clearly lower than the change in IgG4 observed by Durham et al. [18] who investigated IgG4 induction with SLIT tablet in more than 100 patients treated with grass SLIT tablets. However, both reports indicate a fast onset of the blocking effect after around 3 months' treatment and for IgE-blocking factor clearly mirrored the changes in IgG4 in both studies.

For SCIT, the changes in both $\log_{10}$ of IgG4 and IgE-blocking factor were about twice that of AIT, in accordance with suggestions from Scadding et al. [21] and in line with general findings in double-blind placebo-controlled (DBPC) trials [17, 18].

Facilitated antigen presentation

Enhanced T-cell activation was observed in the presence of allergen-specific IgE as a result of CD23-dependent IgE-facilitated allergen presentation [24]. This mechanism could not be significantly inhibited at any time point by serum from the control group. However, in the SCIT and SLIT-tablet group, the inhibition of FAP was significantly different from the control group after one and 3 months of treatment, respectively. This early inhibitory effect confirms the findings of several other studies [17, 24, 35, 37]. Interestingly, although the initial rise in FAP inhibition in the SCIT group was significantly higher than the changes induced by SLIT-tablet treatment, this difference disappeared after 12 months of treatment (Fig. 4). This is in contrast to both the IgG4 levels and the IgE-blocking factor values which remained significantly lower in the SLIT-tablet group.

Basophil sensitivity

The decreased basophil sensitivity to allergen challenge following 6 months' SLIT-tablet treatment is a new finding, whereas the decrease following SCIT treatment is well known [38–40]. A recent paper by Swamy et al. [41] reports that significantly decreased allergen-specific basophil reactivity was observed after 2 years of dual sublingual immunotherapy (SLIT) grass and mite immunotherapy, while van Overtvelt et al. [42] report that no effect on basophil sensitivity was seen after 4 months of SLIT-tablet treatment. Although slightly different BAT readouts were used, our data seem to be

in accordance with both findings, as we showed that basophil sensitivity was significantly decreased from 6 months' treatment and continued to decrease with prolonged treatment. Probably, the decreased sensitivity is caused by a combination of humoral [19, 39, 43, 44] and cellular [45] changes.

The treatment-dependent differences in humoral and cellular assays could theoretically be related to cumulative allergen exposure. The allergen activity units used for SCIT and SLIT tablets are not completely comparable. However, the content of the major allergen Phl p 5 is identical (10 000 units = 2 μg of Phl p 5 allergen [46]) so this major allergen may be used to align the treatment doses. Treatment with SLIT tablet is constant (15 μg of Phl p 5 daily throughout the study), whereas SCIT treatment is administered much slower initially, and not constant until after 3 months of treatment (maintenance dose is 20.0 μg of Phl p 5 every 2 months, corresponding to about 0.2 μg daily). Consequently, the cumulative dose of Phl p 5 the first 4 months of treatment is about 85.0 μg for SCIT and 1800.0 μg for SLIT-tablet treatment. Towards the end of the study, the SCIT group had received about 285.0 μg of Phl p 5, whereas the SLIT-tablet group had received about 6300.0 μg of Phl p 5. From our perspective, these differences in allergen exposure can by no means explain the immunological changes observed as often reported in the literature when links between immunological effects and accumulative doses are sought [47], and therefore, we would assume that the immunological effects are strongly influenced by the routes of administration (subcutaneously vs. sublingually) as well as by the allergen formulation as ALK Alutard contains aluminium known to boost antibody production [48], whereas ALK Grazax does not.

Mucosal uptake is likely to induce additional immunological changes not investigated in the present study. Other subclasses of antibodies and/or mediators possibly play a role in inhibiting the activation of mast cells and basophils. Durham and colleagues argue that the mucosal application of allergen may induce local regulatory mechanisms involving T cell–B cell interaction which in turn promote a greater proportion of high-affinity IgG4 more capable of competing with IgE for binding to allergen [18]. Our data show comparable correlations between IgE-blocking factor and IgG4 for the two treatment groups ($R = 0.69$ and $R = 0.64$ for SLIT and SCIT, respectively) and that fold increase in IgG4 significantly influences IgE-blocking factor, suggesting that the increases in IgG4 titres and in IgE-blocking factor are closely linked for both treatments. Interestingly, however, a significant correlation between FAP and IgG4 was observed for the SLIT group only. This finding could suggest that SLIT-tablet treatment activates mechanisms that increase IgG4 blocking capacity when cellular receptors are involved, while

SCIT does not. However, lack of significant correlations or interactions may merely reflect the modest group sizes in this study. In general, our data indicate that SLIT tablets and SCIT activate similar immunological elements but suggest that the link between the different antibody-mediated changes may be more complex for treatments based on mucosal uptake, as previously suggested by Shamji et al. and Scadding et al. [14, 21].

In addition, changes in local T-cell cytokine responses and inflammatory mediators could possibly reveal novel effects of mucosal application of immunotherapy. We attempted to address this issue in the current study using procedures developed by Scadding et al. [27], where symptoms and nasal secretion were recorded after controlled nasal exposure to grass pollen. Inconsistency in the collection of nasal secretion samples made it impossible to make a time course analysis of the immunological parameters. Nasal challenge total symptom scores were collected based on self-reported symptoms after a non-blinded challenge. The observed difference in symptom score between SLIT tablet and SCIT is in contrast to the reported similarity in clinical effect [13], suggesting that the nasal challenge needs further optimization/validation to reflect the symptoms experienced after *in vivo* pollen exposure.

Based on registry data, it has been shown that those who respond well to SCIT do so within the first year of treatment [49], and within this timeframe, moderate correlations between the clinical effect and changes in blocking antibodies and FAP have previously been demonstrated for SCIT [17]. The immunological findings in this study indicate that such correlations should be sought separately for individuals treated with SLIT tablets. In addition, even though systemic immunological changes could be indicative of the general effect of SLIT tablets, other more local immunological effects should be considered when seeking biomarkers directly reflecting the clinical effect of SLIT-tablet treatment. And although we demonstrated an acceptable adherence level (89%) in the SLIT-tablet group, adherence could have been even lower, because participants took their medication at home. Finally, because the pollen season affected the laboratory measures, especially for specific IgE and BAT, these measurements may also have been affected by the nasal challenges. It has been shown that nasal challenge with ragweed induced an increase in serum-specific IgE, and the levels remained elevated for 8 months [50], but our data do not support these findings.

Future perspectives

Changes in cellular reactivity and distribution of different T-cell subsets have been observed during both SCIT and SLIT tablets [14–16]. Peripheral blood mononuclear cells (PBMCs) collected during the present study have been stored for future experiments addressing shifts in T-cell cytokine production and Treg functionality. In addition, possible links between antibody-mediated and cellular changes both during and after treatment with immunotherapy may be investigated.

Conclusions

Both SLIT tablet and SQ-standardized SCIT induced significant immunological changes that can be observed in both humoral (IgE, IgE-blocking factor, and IgG4) and cellular (facilitated antigen presentation and basophil sensitivity) assays. When comparing SLIT tablet with SCIT, the humoral changes are generally similar, although SCIT induced much larger (two- to threefold) changes, except for specific IgE where SLIT tablet induced a larger initial response. SCIT induced a fast onset in FAP inhibition, whereas SLIT tablet a more gradual increase reaching levels not significantly different from SCIT after 10 months of treatment. Basophil sensitivity indicated the same overall trend as FAP, although 15 months' treatment was not sufficient time to completely eradicate the differences between SLIT-tablet treatment and SCIT.

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Conflict of interest

The Respiratory Research Unit at Bispebjerg University Hospital has received financial support for their research from ALK (Hørsholm, Denmark).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. CONSORT diagram and description of Basophil activation test (BAT).

Immunological comparison of allergen immunotherapy tablet treatment and subcutaneous immunotherapy against grass allergy

Authors

Kristian Aasbjerg, MD (1)
Vibeke Backer, MD, DMSc (1)
Gitte Lund, Research Scientist (2)
Jens Holm, PhD (2)
Niels Christian Nielsen, MSc (2)
Mille Holse, PhD (2)
Valery Renard Wagtmann, PhD (2)
Peter Adler Würtzen, PhD (2)

Affiliations

1) Respiratory Research Unit, Department of Lung Medicine
Copenhagen University Hospital Bispebjerg,
Copenhagen
Denmark

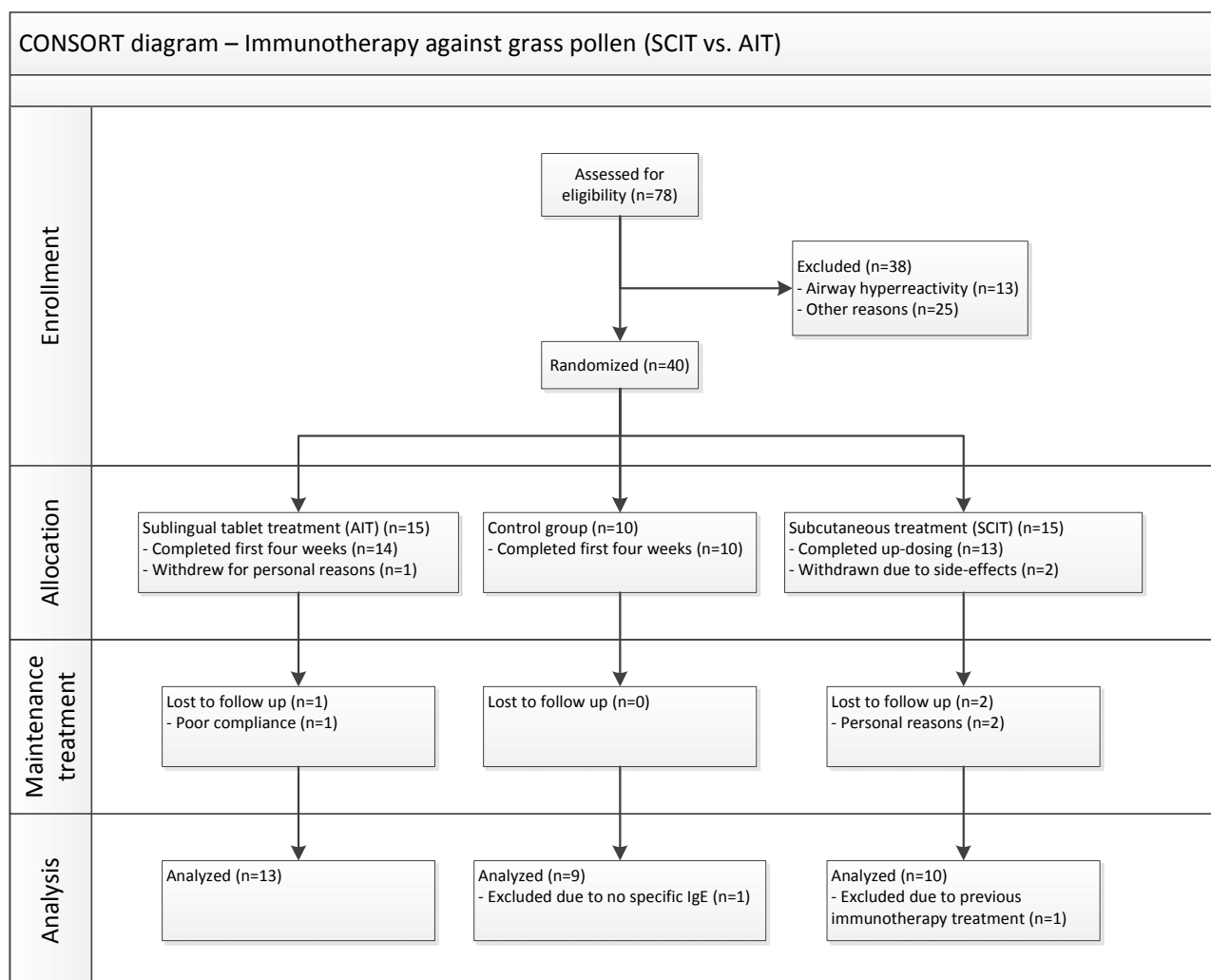
2) ALK
Hørsholm
Denmark

Correspondence

Kristian Aasbjerg (MD)
Respiratory Research Unit, Department of Lung Medicine
Copenhagen University Hospital Bispebjerg, Entrance 66
DK-2400 Copenhagen NV
E-mail: kristian@aasbjerg.dk
Mobile: +45 4072 6210
Phone: +45 3531 3569
Fax: +45 3531 2179

3 Supplemental material

CONSORT diagram



Basophil activation test (BAT)

A widely used strategy for addressing basophil activation based on CD63 expression is to use an activation gating based on 1% of the basophil population in resting cells. We discarded this method as we have observed high differences in activation level in resting cells between patients and time points and assume that this may cause unwanted variation in the readout. We therefore

used a strategy where we defined an activation gate based on a population of highly activated cells in each experiment, and defined the number of basophils in this gate as “degranulated cells”. CD203c and CD63 expression were also changed in cells outside this gate and in order to address this, we also calculated mean expression of CD63 and CD203c respectively in the whole basophile population.

This strategy results in 3 values from each sample: % degranulation, CD63 MF, CD203c MF. In the current paper we have included EC50 values based on % degranulation data, however comparable conclusions were found if we used EC50 values calculated by either CD63 mean fluorescence or CD203c mean fluorescence.

Basophils were identified based as CCR3 and CD203c db positive cells combined with SSC. In contrast to a widely used approach for basophil gating based on low SSC we also included cells with high CD203c and high SSC in the basophile population (Fig x), this was due to activation induced increased SSC in a large group of the patients and we found this gating necessary in order to include all basophils.

In brief basophil activation was performed by incubation of 100 µl heparinized whole blood with different doses of *P. pratense* for 1 h. In parallel activation with and without priming with 2ng/ml IL-3 were made. In the current paper we have included data from EC50 values calculated from BAT without priming, however comparable conclusions were found using a protocol with IL-3.

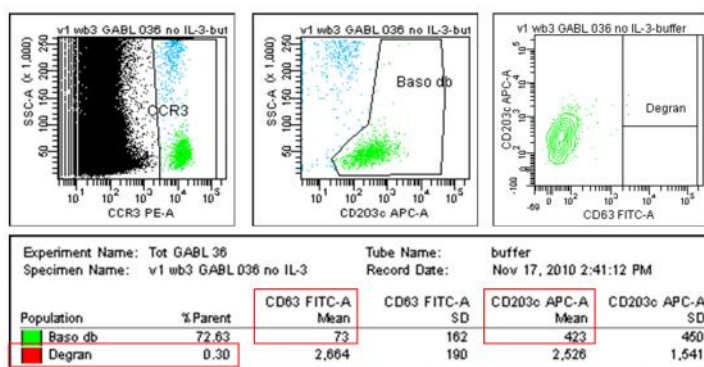
FACS plot examples showing gating and comparison strategies used.

- Basophils were identified based as CCR3 and CD203c db positive cells.

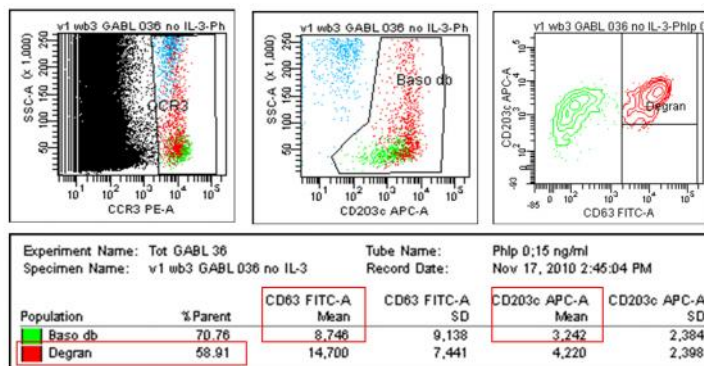
- A gate for calculating highly degranulated cells were made based on maximal activated samples.
- CD63 and CD203c mean fluorescence expression for the whole basophile population were measured.

Whole blood activation : 3 output from each sample: % degranulation, CD63 MF, CD203c MF

No activation
(buffer)



Activation
(Phl p)



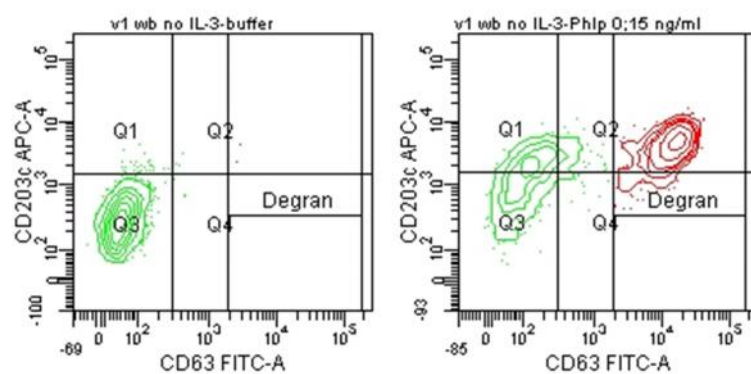


Table 1: Summary of immunology. Significant changes are marked.

Parameter	Treatment months (0 = treatment start and -3 at time of enrolment)	Control	AIT	SCIT
<u>ΔLog(IgE to grass (Phleum pratense)kU/L)</u>	-3	0.00 (-0.13-0.13)	0.00 (-0.10-0.10)	0.00 (-0.10-0.10)
	0	0.11 (-0.02-0.23)	0.11 (0.00-0.21)	0.06 (-0.04-0.16)
	1	0.06 (-0.06-0.19)	0.63 (0.53-0.73)H	0.08 (-0.03-0.18)†
	2	0.05 (-0.07-0.18)	0.63 (0.53-0.73)H	0.21 (0.11-0.31)H,†
	3	0.02 (-0.10-0.15)	0.58 (0.48-0.68)H	0.21 (0.11-0.31)H,†
	6	0.33 (0.20-0.46)§	0.55 (0.44-0.65)H	0.14 (0.03-0.24)H,†
	7	0.28 (0.15-0.40)§	0.48 (0.38-0.58)H	0.09 (-0.02-0.20)H,†
	10	0.17 (0.04-0.30)§	0.40 (0.29-0.50)H	0.04 (-0.07-0.15)†
	12	0.20 (0.07-0.32)§	0.30 (0.20-0.41)	-0.03 (-0.14-0.08)H,†
	15	0.07 (-0.06-0.19)	0.20 (0.09-0.30)	-0.04 (-0.15-0.07)†
<u>ΔLog(IgG4 (mgA/L))</u>	-3	0.00 (-0.21-0.21)	0.00 (-0.16-0.16)	0.00 (-0.16-0.16)
	0	-0.03 (-0.24-0.18)	-0.01 (-0.17-0.14)	0.00 (-0.15-0.16)
	1	-0.02 (-0.23-0.18)	0.24 (0.08-0.39)H	0.20 (0.04-0.35)
	2	-0.04 (-0.25-0.16)	0.33 (0.17-0.48)H	0.76 (0.60-0.91)H,†
	3	-0.05 (-0.26-0.15)	0.42 (0.26-0.57)H	1.27 (1.11-1.42)H,†
	6	0.10 (-0.10-0.31)	0.51 (0.36-0.67)H	1.39 (1.23-1.55)H,†
	7	0.10 (-0.10-0.31)	0.55 (0.39-0.70)H	1.45 (1.29-1.61)H,†
	10	0.04 (-0.17-0.26)	0.55 (0.39-0.70)H	1.53 (1.37-1.70)H,†
	12	0.00 (-0.20-0.21)	0.57 (0.41-0.73)H	1.51 (1.34-1.68)H,†
	15	-0.02 (-0.23-0.18)	0.60 (0.44-0.76)H	1.71 (1.54-1.88)H,†
<u>ΔIgE(blocking antibodies)</u>	-3	0.00 (-0.10-0.10)	0.00 (-0.08-0.08)	0.00 (-0.08-0.08)
	0	0.03 (-0.07-0.13)	0.04 (-0.04-0.11)	0.01 (-0.07-0.08)
	1	0.03 (-0.06-0.13)	0.11 (0.03-0.18)	0.02 (-0.05-0.10)
	2	0.02 (-0.08-0.12)	0.13 (0.06-0.21)	0.16 (0.08-0.24)H
	3	0.00 (-0.10-0.10)	0.18 (0.10-0.25)H	0.47 (0.39-0.55)H,†
	6	0.08 (-0.02-0.17)	0.20 (0.12-0.28)H	0.50 (0.42-0.58)H,†
	7	0.06 (-0.04-0.16)	0.23 (0.15-0.31)H	0.50 (0.42-0.58)H,†
	10	0.04 (-0.06-0.14)	0.23 (0.15-0.31)H	0.50 (0.41-0.58)H,†
	12	0.06 (-0.04-0.15)	0.26 (0.18-0.33)H	0.46 (0.37-0.54)H,†
	15	0.04 (-0.05-0.14)	0.27 (0.19-0.35)H	0.56 (0.47-0.64)H,†
<u>ΔFacilitated Antigen Presentation (%inhibition)</u>	-2	0.00 (-4.47-4.47)	0.00 (-3.58-3.58)	0.00 (-3.58-3.58)
	0	-0.60 (-5.07-3.87)	-1.81 (-5.39-1.77)	-1.02 (-4.60-2.57)
	1	-0.58 (-5.05-3.89)	-1.61 (-5.20-1.97)	2.56 (-1.03-6.14)
	2	-0.35 (-4.82-4.12)	2.54 (-1.05-6.12)	13.6 (10.0-17.2)H,†
	3	-1.43 (-5.90-3.04)	6.72 (3.14-10.31)H	22.5 (18.8-26.1)H,†
	6	-1.63 (-6.10-2.84)	10.6 (7.0-14.1)H	21.1 (17.4-24.8)H,†
	7	-0.03 (-4.50-4.44)	11.4 (7.7-15.1)H	22.5 (18.8-26.3)H,†
	10	-2.43 (-7.24-2.39)	12.0 (8.4-15.7)H	20.7 (16.7-24.7)H,†
	12	-2.39 (-6.86-2.08)	16.0 (12.2-19.7)H	20.2 (16.2-24.1)H
	15	-2.68 (-7.15-1.79)	17.2 (13.4-20.9)H	22.0 (18.0-26.0)H
<u>ΔLog(1/Basophil activation test (EC50))</u>	-2	0.00 (-0.27-0.27)	0.00 (-0.21-0.21)	0.00 (-0.23-0.23)
	1	-0.02 (-0.28-0.25)	0.18 (-0.03-0.39)	-0.35 (-0.58--0.12)†
	3	0.15 (-0.11-0.42)	-0.13 (-0.35-0.08)	-1.07 (-1.30--0.84)H,†
	6	0.30 (0.02-0.59)§	-0.17 (-0.40-0.06)H	-1.15 (-1.39--0.90)H,†
	7	0.33 (0.06-0.61)§	-0.10 (-0.31-0.11)H	-1.10 (-1.33--0.86)H,†
	10	0.24 (-0.04-0.51)	-0.45 (-0.66--0.23)H	-1.35 (-1.59--1.11)H,†
	12	0.09 (-0.17-0.36)	-0.67 (-0.88--0.45)H	-1.30 (-1.55--1.06)H,†
	15	0.04 (-0.23-0.31)	-0.71 (-0.93--0.49)H	-1.40 (-1.65--1.16)H,†
<u>ΔTotal symptom score 30 minutes post-nasal challenge</u>	-2	0.00 (-27.60-27.60)	0.00 (-21.38-21.38)	0.00 (-22.13-22.13)
	3	9.11 (-18.49-36.71)	-6.21 (-28.15-15.73)	-55.1 (-77.8--32.3)H,†
	10	-2.32 (-30.97-26.34)	-25.1 (-47.0--3.1)	-52.2 (-76.3--28.0)H
	15	-2.22 (-29.82-25.37)	-32.6 (-55.1--10.1)	-56.8 (-81.7--31.9)H

§ = Control group significantly different from baseline (treatment months = 0).

¤ = Significantly different from control group.

† = Significantly different when compared with AIT.

Immunological comparison of allergen immunotherapy tablet treatment and subcutaneous immunotherapy against grass allergy

Authors

Kristian Aasbjerg, MD (1)
Vibeke Backer, MD, DMSc (1)
Gitte Lund, Research Scientist (2)
Jens Holm, PhD (2)
Niels Christian Nielsen, MSc (2)
Mille Holse, PhD (2)
Valery Renard Wagtmann, PhD (2)
Peter Adler Würtzen, PhD (2)

Affiliations

1) Respiratory Research Unit, Department of Lung Medicine
Copenhagen University Hospital Bispebjerg,
Copenhagen
Denmark

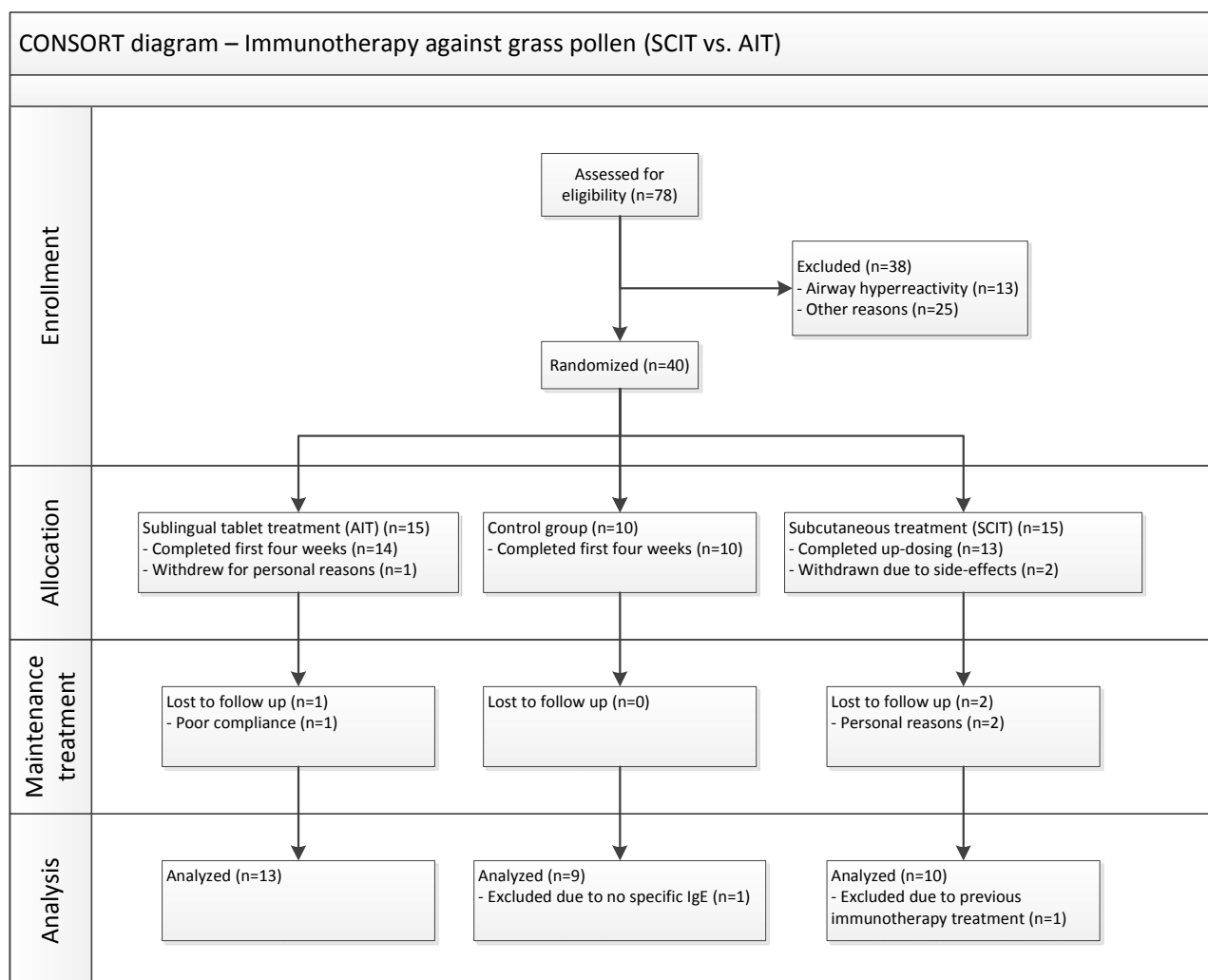
2) ALK
Hørsholm
Denmark

Correspondence

Kristian Aasbjerg (MD)
Respiratory Research Unit, Department of Lung Medicine
Copenhagen University Hospital Bispebjerg, Entrance 66
DK-2400 Copenhagen NV
E-mail: kristian@aasbjerg.dk
Mobile: +45 4072 6210
Phone: +45 3531 3569
Fax: +45 3531 2179

3 Supplemental material

32 CONSORT diagram



34 Basophil activation test (BAT)

A widely used strategy for addressing basophil activation based on CD63 expression is to use an activation gating based on 1% of the basophil population in resting cells. We discarded this method as we have observed high differences in activation level in resting cells between patients and time points and assume that this may cause unwanted variation in the readout. We therefore

used a strategy where we defined an activation gate based on a population of highly activated cells in each experiment, and defined the number of basophils in this gate as “degranulated cells”. CD203c and CD63 expression were also changed in cells outside this gate and in order to address this, we also calculated mean expression of CD63 and CD203c respectively in the whole basophile population.

This strategy results in 3 values from each sample: % degranulation, CD63 MF, CD203c MF. In the current paper we have included EC50 values based on % degranulation data, however comparable conclusions were found if we used EC50 values calculated by either CD63 mean fluorescence or CD203c mean fluorescence.

Basophils were identified based as CCR3 and CD203c db positive cells combined with SSC. In contrast to a widely used approach for basophil gating based on low SSC we also included cells with high CD203c and high SSC in the basophile population (Fig x), this was due to activation induced increased SSC in a large group of the patients and we found this gating necessary in order to include all basophils.

In brief basophil activation was performed by incubation of 100 µl heparinized whole blood with different doses of *P. pratense* for 1 h. In parallel activation with and without priming with 2ng/ml IL-3 were made. In the current paper we have included data from EC50 values calculated from BAT without priming, however comparable conclusions were found using a protocol with IL-3.

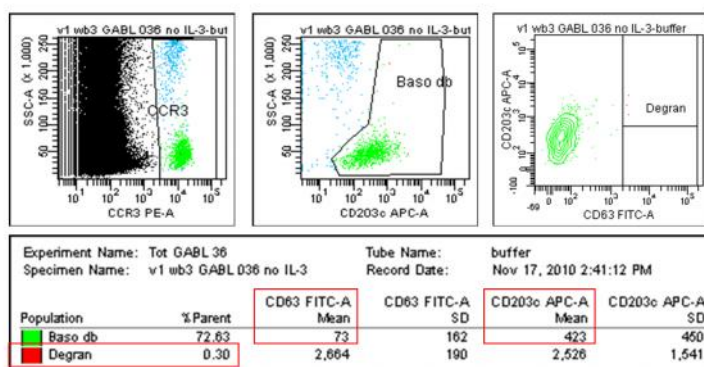
FACS plot examples showing gating and comparison strategies used.

- Basophils were identified based as CCR3 and CD203c db positive cells.

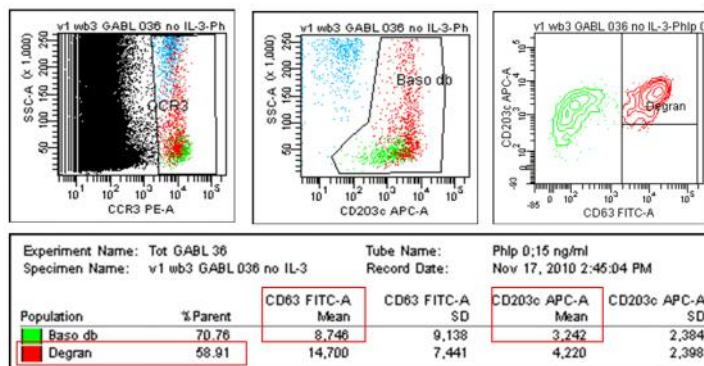
- A gate for calculating highly degranulated cells were made based on maximal activated samples.
- CD63 and CD203c mean fluorescence expression for the whole basophile population were measured.

Whole blood activation : 3 output from each sample: % degranulation, CD63 MF, CD203c MF

No activation
(buffer)



Activation
(Phl p)



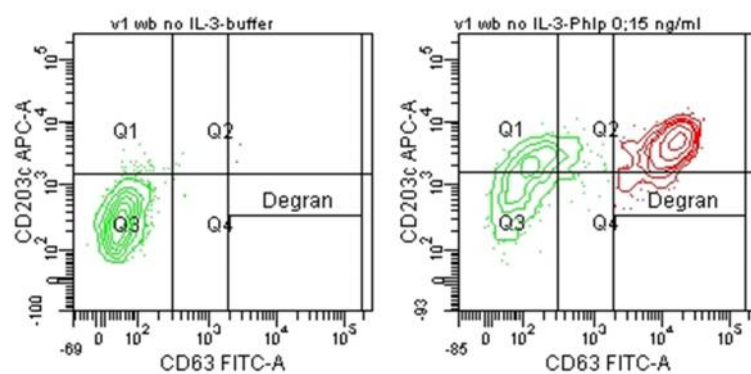


Table 1: Summary of immunology. Significant changes are marked.

Parameter	Treatment months (0 = treatment start and -3 at time of enrolment)	Control	AIT	SCIT
<u>ΔLog(IgE to grass (Phleum pratense)kU/L)</u>	-3	0.00 (-0.13-0.13)	0.00 (-0.10-0.10)	0.00 (-0.10-0.10)
	0	0.11 (-0.02-0.23)	0.11 (0.00-0.21)	0.06 (-0.04-0.16)
	1	0.06 (-0.06-0.19)	0.63 (0.53-0.73)H	0.08 (-0.03-0.18)†
	2	0.05 (-0.07-0.18)	0.63 (0.53-0.73)H	0.21 (0.11-0.31)H,†
	3	0.02 (-0.10-0.15)	0.58 (0.48-0.68)H	0.21 (0.11-0.31)H,†
	6	0.33 (0.20-0.46)§	0.55 (0.44-0.65)H	0.14 (0.03-0.24)H,†
	7	0.28 (0.15-0.40)§	0.48 (0.38-0.58)H	0.09 (-0.02-0.20)H,†
	10	0.17 (0.04-0.30)§	0.40 (0.29-0.50)H	0.04 (-0.07-0.15)†
	12	0.20 (0.07-0.32)§	0.30 (0.20-0.41)	-0.03 (-0.14-0.08)H,†
	15	0.07 (-0.06-0.19)	0.20 (0.09-0.30)	-0.04 (-0.15-0.07)†
<u>ΔLog(IgG4 (mgA/L))</u>	-3	0.00 (-0.21-0.21)	0.00 (-0.16-0.16)	0.00 (-0.16-0.16)
	0	-0.03 (-0.24-0.18)	-0.01 (-0.17-0.14)	0.00 (-0.15-0.16)
	1	-0.02 (-0.23-0.18)	0.24 (0.08-0.39)H	0.20 (0.04-0.35)
	2	-0.04 (-0.25-0.16)	0.33 (0.17-0.48)H	0.76 (0.60-0.91)H,†
	3	-0.05 (-0.26-0.15)	0.42 (0.26-0.57)H	1.27 (1.11-1.42)H,†
	6	0.10 (-0.10-0.31)	0.51 (0.36-0.67)H	1.39 (1.23-1.55)H,†
	7	0.10 (-0.10-0.31)	0.55 (0.39-0.70)H	1.45 (1.29-1.61)H,†
	10	0.04 (-0.17-0.26)	0.55 (0.39-0.70)H	1.53 (1.37-1.70)H,†
	12	0.00 (-0.20-0.21)	0.57 (0.41-0.73)H	1.51 (1.34-1.68)H,†
	15	-0.02 (-0.23-0.18)	0.60 (0.44-0.76)H	1.71 (1.54-1.88)H,†
<u>ΔIgE(blocking antibodies)</u>	-3	0.00 (-0.10-0.10)	0.00 (-0.08-0.08)	0.00 (-0.08-0.08)
	0	0.03 (-0.07-0.13)	0.04 (-0.04-0.11)	0.01 (-0.07-0.08)
	1	0.03 (-0.06-0.13)	0.11 (0.03-0.18)	0.02 (-0.05-0.10)
	2	0.02 (-0.08-0.12)	0.13 (0.06-0.21)	0.16 (0.08-0.24)H
	3	0.00 (-0.10-0.10)	0.18 (0.10-0.25)H	0.47 (0.39-0.55)H,†
	6	0.08 (-0.02-0.17)	0.20 (0.12-0.28)H	0.50 (0.42-0.58)H,†
	7	0.06 (-0.04-0.16)	0.23 (0.15-0.31)H	0.50 (0.42-0.58)H,†
	10	0.04 (-0.06-0.14)	0.23 (0.15-0.31)H	0.50 (0.41-0.58)H,†
	12	0.06 (-0.04-0.15)	0.26 (0.18-0.33)H	0.46 (0.37-0.54)H,†
	15	0.04 (-0.05-0.14)	0.27 (0.19-0.35)H	0.56 (0.47-0.64)H,†
<u>ΔFacilitated Antigen Presentation (%inhibition)</u>	-2	0.00 (-4.47-4.47)	0.00 (-3.58-3.58)	0.00 (-3.58-3.58)
	0	-0.60 (-5.07-3.87)	-1.81 (-5.39-1.77)	-1.02 (-4.60-2.57)
	1	-0.58 (-5.05-3.89)	-1.61 (-5.20-1.97)	2.56 (-1.03-6.14)
	2	-0.35 (-4.82-4.12)	2.54 (-1.05-6.12)	13.6 (10.0-17.2)H,†
	3	-1.43 (-5.90-3.04)	6.72 (3.14-10.31)H	22.5 (18.8-26.1)H,†
	6	-1.63 (-6.10-2.84)	10.6 (7.0-14.1)H	21.1 (17.4-24.8)H,†
	7	-0.03 (-4.50-4.44)	11.4 (7.7-15.1)H	22.5 (18.8-26.3)H,†
	10	-2.43 (-7.24-2.39)	12.0 (8.4-15.7)H	20.7 (16.7-24.7)H,†
	12	-2.39 (-6.86-2.08)	16.0 (12.2-19.7)H	20.2 (16.2-24.1)H
	15	-2.68 (-7.15-1.79)	17.2 (13.4-20.9)H	22.0 (18.0-26.0)H
<u>ΔLog(1/Basophil activation test (EC50))</u>	-2	0.00 (-0.27-0.27)	0.00 (-0.21-0.21)	0.00 (-0.23-0.23)
	1	-0.02 (-0.28-0.25)	0.18 (-0.03-0.39)	-0.35 (-0.58--0.12)†
	3	0.15 (-0.11-0.42)	-0.13 (-0.35-0.08)	-1.07 (-1.30--0.84)H,†
	6	0.30 (0.02-0.59)§	-0.17 (-0.40-0.06)H	-1.15 (-1.39--0.90)H,†
	7	0.33 (0.06-0.61)§	-0.10 (-0.31-0.11)H	-1.10 (-1.33--0.86)H,†
	10	0.24 (-0.04-0.51)	-0.45 (-0.66--0.23)H	-1.35 (-1.59--1.11)H,†
	12	0.09 (-0.17-0.36)	-0.67 (-0.88--0.45)H	-1.30 (-1.55--1.06)H,†
	15	0.04 (-0.23-0.31)	-0.71 (-0.93--0.49)H	-1.40 (-1.65--1.16)H,†
<u>ΔTotal symptom score 30 minutes post-nasal challenge</u>	-2	0.00 (-27.60-27.60)	0.00 (-21.38-21.38)	0.00 (-22.13-22.13)
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