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Simplified AIT for allergy to several tree pollens - Arguments from the immune outcome analyses following treatment with SQ tree SLIT-tablet

Running title: Clinical cross-protection of SQ tree SLIT-tablets confirmed by immunological cross-reactivity

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Author contributions:

PAW and HI contributed to study design, analyzed and interpreted the data, and drafted the manuscript. PMG, and GL performed experiments and analyzed data. TB, SG, and PSA supervised the study and contributed to study design and interpretation of data. All authors contributed to and approved the final version of the manuscript and take responsibility for the manuscript content.

Conflict of interest:

TB is an advisory board member for and has received speaker's fees from ALK, Novartis and Mylan. HI is a consultant for ALK. PAW, PMG, GL, SG, and PSA, are employees of ALK.

Data availability statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

ABSTRACT

Background: The SQ tree SLIT-tablet (containing birch extract) proved clinically significant effects during the pollen season for birch as well as alder/hazel. Immune outcomes of this treatment for allergens from multiple birch homologous trees needs further investigation. We hypothesize that birch pollen extract AIT modulate a highly cross-reactive immune response and that this may be the basis for the observed clinical cross-protection.

Methods: Blood samples were collected from 397 birch allergic patients during SQ tree SLIT-tablet or placebo treatment (1:1) for up to 40 weeks. Serum-IgE and IgG₄ specific to birch, and birch homologous tree pollens from alder, hazel, hornbeam, beech, and chestnut were measured by ImmunoCAP. IgE-Blocking Factor (IgE-BF) for alder, birch, and hazel during treatment were measured by Advia Centaur and blocking effects for birch and all these birch homologous tree pollens were further investigated by basophil activation (BAT). Antibody readouts were investigated in patient subsets. T-cell responses (proliferation) to allergen extracts and peptide pools (group 1 allergens) were investigated in T-cell lines from 29 untreated birch pollen allergic individuals.

Results: Significant Pearson correlations between serum-IgE toward birch, alder, hazel, hornbeam, and beech were observed (r -values >0.86). T-cell reactivity was observed throughout the birch homologous group. Almost identical kinetics for changes in IgE towards birch, alder, and hazel were observed during treatment and similar species-specific changes were seen for serum-IgG₄. IgG₄ reactivity toward birch and alder, hazel, hornbeam, and beech, correlated significantly at end-of-treatment (r -values >0.72). Treatment resulted in similar IgE-BF kinetics for alder, birch, and hazel and blocking of BAT for multiple trees in most actively treated patients investigated.

Conclusions:

Systematic analyses of T-cell and antibody cross-reactivities before and during birch pollen extract AIT provides the immunological basis for the observed clinical effect of SQ tree SLIT-tablet treatment of tree pollen allergy induced by multiple trees in the birch homologous group.

Key Words

Allergy immunotherapy (AIT), SLIT-tablet, birch homologous group, cross-reactivity, immunological mechanism, IgE, IgG₄, T-cells

Abbreviations

AIT	Allergy immunotherapy
Alh i/g	<i>Alnus incana</i> /glutinosa (alder)
BAT	Basophil activation test
Bet v	<i>Betula verrucosa</i> /pendula (birch)
Cas s	<i>Castanea sativa</i> (chestnut)
Cor a	<i>Corylus avellana</i> (hazel)
Car b	<i>Carpinus betulus</i> (hornbeam)
EEC	Environmental exposure chamber
FAB	Facilitated allergen binding
Fag g/s	<i>Fagus grandifolia</i> /sylvatica (beech)
IgE-BF	IgE blocking factor
LLQ	Lower level of quantification
LME	Linear mixed effects model
MS	Mass spectrometry
PBMC	Peripheral blood mononuclear cells
PHA	Phytohaemagglutinin
SI	Stimulation Index
PR-10	Pathogenesis-related protein-10
Que a	<i>Quercus alba</i> (oak)
RC	Recoded
IgE	IgE
IgG ₄	IgG ₄
SLIT-tablet	Sublingual allergy immunotherapy tablet
SQ	Standard Quality

Introduction

In Europe and North America, exposure to pollen from birch related trees is a common cause of respiratory allergic diseases with a prevalence of 1.5-17.5% in 13 developed countries worldwide¹. Five tree species (birch/Bet v, alder/Aln g, hornbeam/Car b, hazel/Cor a, and oak/Que a) have been assigned to the same homologous group² termed the "birch homologous group" and additional trees may be included when more knowledge becomes available³. Bet v 1 is the major allergen in birch^{4,5} and various birch related trees contains PR-10 like molecules with high sequence identity to Bet v 1^{6,7,8,9,10} and very similar tertiary structures^{11,12}. Several minor allergens are also shared between some or all of the birch homologous tree species¹³, and birch has been suggested as the dominant common denominator based on IgE cross-reactivity and inhibition/depletion studies^{5,14}. Moreover, the cross-reactivity towards Bet v 1 and its homologous allergens from alder, hazel, hornbeam, and oak has been demonstrated for T-cell lines and clones from individual birch pollen allergic patients^{15,16,17,18}. All of these findings are the foundation for the definition of the birch homologous group of trees². In accordance with this European and US guidelines states that cross-reactive or homologous species should be represented by one member of the individual groups of "cross-reactive or homologous species" for allergy diagnosis and AIT treatment^{19,20}. A recent trial on the SQ tree SLIT-tablet containing *Betula verrucosa* allergen extract further substantiated this for tree pollen allergic patients, where symptom relief was observed when exposing patients to birch as well as to oak pollen in an environmental exposure chamber (EEC)²¹. An in depth analysis of the immune outcome in regard to effects across the birch homologous group following allergen-specific immunotherapy with birch pollen extracts is still missing. As a result of the IgE and T-cell cross-reactivity, most patients who are allergic to birch pollen experience symptoms when exposed to pollen from the other members of the birch homologous group, which increases the disease burden in terms of season duration and relevant regions²².

The changes in the allergen-specific immune response during AIT with allergen extracts includes induction of blocking non-IgE antibodies, as well as shifting the balance between Th1/Th2 and regulatory T-cells²³. Thus, recognition of specific T- and B-cell epitopes appears to play a key role in mediating clinical effect towards the allergen extract used for treatment or the allergens recognized through cross-reactivity. This makes it important to demonstrate immunological cross-reactivity within closely related species; not only for causing allergic symptoms but also for securing clinical benefit of AIT towards symptoms induced by closely related allergen sources. In fact, monitoring the immunological changes induced by birch pollen specific immunotherapy could allow for identification of early markers for effectiveness not only for birch pollen allergy, but within the whole birch homologous group based on immune cross-protection. Indeed,

comparable changes in serum IgE and IgG₄ specific to birch and to oak accompanied reductions in rhinitis symptoms during EEC allergen challenge, further supporting that clinical cross-protection is linked to immunological cross-reactivity. Moreover, in the pivotal phase III trial investigating the clinical efficacy of the SQ tree SLIT-tablet, significant and clinically relevant reductions in symptom and medication scores were observed during the birch pollen season, the combined alder/hazel and birch pollen seasons as well as these seasons investigated separately, again confirming the cross-protection induced by AIT with a representative species²⁴. In the current investigation, cross-reactivity of IgE as well as T-cells was characterized in detail and the previously reported changes induced by the tree-SLIT tablet in IgE and IgG₄ towards birch²⁴ were supplemented by analyzing the immunological cross-reactivity of serum IgE and IgG₄ towards multiple trees within the birch homologous group and addressing the functionality of these antibody changes in IgE-BF and basophil activation test (BAT) experiments.

Methods

Design:

The clinical trial is identified by ClinicalTrials.gov Identifier EudraCT 2015-004821-15 and details of the trial design and patient demographics were reported previously²⁴. Briefly, this was a randomized, parallel-group, double-blind, placebo-controlled, multi-site, phase III field trial with 1:1 randomization between treatment with SQ tree SLIT-tablet or placebo. Subjects were included on basis of a positive skin prick test response (wheal diameter ≥ 3 mm) to birch (*Betula verrucosa*) extract, a positive Bet v 1 specific IgE ($\geq$ IgE Class 2; ≥ 0.7 kUA/L) and moderate to severe allergic rhinoconjunctivitis symptoms during the 2 previous birch pollen seasons. Blood samples were collected from 397 subjects at baseline and after 1, 4, 7, and 9 months of treatment. Collection of blood samples were approved by the local ethics committees approving the trial (EudraCT 2015-004821-15) and informed consent were obtained from all subjects donating blood for the immunological tests.

The immune outcomes were investigated to address:

- 1) Cross-reactivity of IgE (n=397, baseline) and T-cells (n=29, untreated) involved in the establishment of an allergic sensitization towards multiple birch homologous group pollen allergens.
- 2) Change in IgE and IgG₄ specific to birch homologous group pollen allergens (birch, alder, and hazel) induced by tree SLIT-tablet treatment (n=200, at baseline and after 1, 4, 7, and 9 months of treatment).
- 3) The functional effect of these changes investigated by IgE-blocking factor, which reflects the competition between allergen-specific IgE and treatment induced allergen-

specific "non-IgE" antibodies (IgG₄ and other isotypes apart from IgE) for binding to the allergen (n=160-200).

- 4) Cross-reactivity of treatment induced IgG₄ to multiple birch homologous group pollen allergens (birch, alder, hazel, hornbeam, beech, and chestnut, n=282, end of treatment).
- 5) The functional effect of these changes for multiple birch homologous group pollen allergens was exemplified by basophil activation test (passive sensitization), which reflects how the competition between allergen-specific IgE and treatment induced allergen-specific "non-IgE" antibodies influence allergen-specific activation of effector cells (n=19).

Serum antibodies and IgE-blocking factor analyses

100 SQ tree SLIT-tablet and 100 placebo treated subjects from the phase III trial were selected at random for IgE, IgG₄ and IgE-BF analyses.

At baseline and after 1, 4, 7, and 9 months of treatment, serum samples were analysed for Bet v, Aln i, and Cor a-specific IgE and IgG₄ antibodies by ImmunoCAP (Phadia 250, Thermo Fischer Scientific, Uppsala, Sweden) according to the manufacturers instructions and Bet v IgE-BF was measured as described previously²⁵. Similar assays were implemented for Aln g and Cor a IgE-BF with standard Advia Centaur reagents.

For antibody cross-reactivity studies, serum specific IgE (*pre-treatment*, n=397, *placebo:active* = 1:1) and IgG₄ (*post-treatment*, n=282, *all actively treated*) to birch (Bet v), hazel (Cor a), alder (Aln i), hornbeam (Car b), beech (Fag g), and chestnut (Cas s) were measured by ImmunoCAP. Values below lower limit of quantification (LLQ) for IgE (LLQ=0.7 kUA/L) and IgG₄ (LLQ=0.15 mgA/L) was given the value LLQ/2.

BAT

Basophile activation assay, BAT was performed with passively sensitized basophils from non-atopic donors. Freshly isolated PBMC were stripped for IgE by treatment with acetic acid and subsequently sensitized with pre-IT serum from the individual patients as described by Kleine et al.²⁶. The allergen preparations (0.25 ng/ml to 250 µg/ml) were diluted in RPMI 0,5 % HSA, mixed with pre or post-IT sera (10%) and incubated with sensitized basophils (PBMC) at 37 degrees for 60 min. After incubation, cells were washed and stained with CD123-PE (BD Bioscience, San Jose, CA) and CD203c_APC (Biolegend, San Diego, CA) for identification of basophils and CD63-FITC (BD Bioscience, San Jose, CA) for measuring activation by FACS

analysis. Cells were analysed on Fortessa flowcytometer, data analysed using FLOW-Jo software to generate dose-response activation curves and calculation of EC50 using Graph Pad Prism. All samples from the same patient, before/after immunotherapy and activated with extracts from different pollens were analysed in the same experiment.

T-cell analyses

PBMC from 29 birch pollen allergic patients (Bet v IgE > 0.7 kU/L, ImmunoCAP). Bet v-specific T-cells from cryopreserved PBMC (Danish ethics committee approval H-3-2014-129) were expanded *in vitro*, and specific responses were measured by FluoroSPOT²⁷ on day 14 when cell counts allowed and by thymidine incorporation²⁷ on day 24 or later. In these assays, the T-cell lines were stimulated with peptide pools (2 µg/mL), allergen extracts (5-10 µg/mL), or controls (PHA or medium alone).

Criteria for positive responses were set as previously described²⁷ on basis of significant positive responses (Student's t-test, $p < 0.05$) and stimulation index (SI) > 2 for fluoroSPOT and SI > 3 for frequencies in proliferation assays. Data are represented as % of the stimulation observed for Bet v extract to normalize the data relative to the extract used to initiate the T-cell lines.

Allergen extracts and peptides:

Experimental extracts of Bet v, Aln g, Cor a, Car b, Fag s, and Cas s were made as follows:

Pollen from each species (10 g) was extracted in 100 mL of NH_4HCO_3 (0.125 mol/L, pH 8.3) at 5°C for 2 hours followed by dialysis (MW cut-off 3.5 kD), filtration (0.2 µm), and freeze-drying.

20mers peptides (overlapping by 10 aa) covering amino acid sequences of Bet v 1, Aln g 1, Cor a 1, Car b 1, Fag s 1, and Cas s 1 were custom-made by Genscript NJ, USA with a purity of ≥ 95%.

The species of alder and beech differs between ImmunoCAP assays and T-cell experiments because Aln i and Fag g are the species available for ImmunoCAP whereas group 1 major allergen sequences of Aln g and Fag s were reported in the literature and annotated in IUIS databases. Endotoxin content of all extracts were below 20 EU/mg except Cor a and Cas s which were below 100 EU/mg.

Allergen sequences selected for peptide design

Bet v 1.112 (or Bet v 1.2801) is a well characterized Bet v 1 isoform with data available for the crystal structure of the molecule alone or in complex with a Bet v 1-specific antibody^{11,12,28} and high abundance of this isoform was confirmed by mass spec (MS) analysis. Aln g 1.0101 is the only IUIS aa sequence available for this tree species. Cor a extract was analysed by mass spec

where Cor a 1.0102 and 1.0103 were the two most abundant isoforms identified and Cor a 1.0102 was selected for design of Cor a 1 peptides.

Car b 1.0109 was selected for peptide design based on data from wallner et al demonstrating that Car b 1.0109 is among the most abundant Car b 1 isoforms in this tree pollen allergen extract ²⁹. Cas s 1.0101 and Fag s 1.0101 are the only IUIS aa sequences available for these allergens. All cysteines residues were replaced by serine in the 20-mer peptides to avoid cross-linking.

Statistical methodology

Normality was assessed by visual inspection of normal quantile plots of the data (log10 transformed for IgE and IgG₄ measurements). The change from baseline was assessed by fitting to a linear mixed model using subjects as random factor assuming compound symmetry. Post hoc comparisons of all pairs were performed using Tukey's HSD (i.e. a single-step multiple comparison procedure and statistical test). The least square mean values were calculated from the model and plotted with 95% confidence limits after back transformation to obtain fold change values. IgE and IgG₄ cross-reactivity was analysed by Pearson correlations for all samples with quantifiable serum concentrations binding to Bet v as well as the homologous tree pollen allergen extract. The percentage of samples binding to Bet v allergen extract as well as the individual homologous tree pollen extract is indicated as well for all correlations analysed. All calculations were performed in SAS JMP version 13.2 or later. Changes in basophil sensitivity (BAT, EC50) were investigated by wilcoxon ranked sum test using Graph Pad Prism. Differences in T-cell responses were evaluated by Friedman nonparametric ANOVA and Dunns multiple comparison Rank sum test using Graph Pad Prism.

Results

IgE changes during treatment

Serum levels of allergen-specific IgE were analysed to investigate how SQ tree SLIT-tablet treatment modulates the existing allergen-specific immune response. As shown in Figure 1 (and online figure E4 for data from individual subjects), an initial induction of allergen-specific IgE was seen for alder, birch, and hazel peaking at 4 weeks after initiation of treatment with an approximate 3-fold increase. This was followed by a decrease during the remaining treatment period for the 3 tree pollen allergens investigated. At the end of treatment, allergen-specific IgE was still significantly increased in the SQ tree SLIT-tablet group compared to placebo, even though the serum concentrations for the two treatment groups were approaching the same level.

IgG₄ changes during treatment

Induction of allergen-specific IgG₄ is a hallmark of AIT and seen as an indicator of the competing non-IgE antibody response induced by AIT. A significant induction of allergen-specific IgG₄ was observed in the current trial for alder, birch, and hazel after 4 weeks of treatment (Figure 2 and online figure E5 for data from individual subjects). The serum IgG₄ concentrations increased further until 4 months of treatment, followed by a slight further increase for birch and a plateau for alder and hazel. The max increase during the trial for birch was 4-5 fold whereas a 2-3 fold increase was observed for alder and hazel.

IgE-Blocking Factor induction during treatment

The IgE-BF reflects the competition between allergen-specific IgE and treatment induced non-IgE antibodies and this assay is used to address the functionality of the quantitative changes seen for allergen-specific IgE and IgG₄. The data depicted in figure 3 demonstrate a significant induction of IgE-BF after 4 weeks of treatment for the three tree pollen allergen extracts investigated. The inhibitory effect was further increased until 4 month of treatment, followed by a plateau towards the end of treatment. The quantitation of the blocking effect may not be fully comparable between these three assays, however, strongest blocking effect was observed for birch, followed by alder and with hazel showing slightly delayed induction with optimal level after 7 month of treatment.

Cross-reactivity of pre-treatment serum IgE.

Sensitization towards closely related tree species may be the result of cross-reactivity of IgE antibodies. Clear correlations (Pearson) between serum IgE concentrations specific to different trees indicate cross-reactivity. IgE sensitisation and the correlation between IgE titers towards individual trees of the birch homologous group is shown in Figure 4. The majority of the birch allergic patients had serum IgE binding to multiple related trees ranging from 95% (beech) to 99% (alder), whereas only 14% reacted to chestnut. The data demonstrate significant correlations between IgE reactivity towards birch and alder, hazel, and hornbeam (birch homologous group species) ($r > 0.93$) as well as beech ($r = 0.93$). The strongest correlation in IgE titers was seen between birch and alder ($r = 0.98$) and the weakest was between birch and hazel or beech ($r = 0.93$). There was no correlation between birch and chestnut ($r = 0.047$).

Cross-reactivity of end-of-treatment serum IgG₄

Cross-reactivity of IgG₄ indicates that treatment induced immune modulation affects the response to different closely related tree species. This was investigated by analysis of correlations similar

to the analyses of IgE sensitization. The correlation between IgG₄ serum concentrations towards birch and serum IgG₄ concentrations towards the individual trees of the birch homologous group is shown in Figure 5. Serum from the majority of the patients treated with SQ Tree SLIT-tablets contained IgG₄ binding to multiple related trees ranging from 80% (birch/hornbeam) to 90% (birch/alder), whereas only 8% contained IgG₄ towards chestnut in addition to birch. Significant correlation were found between IgG₄ towards birch and alder, hazel, and hornbeam (birch homologous group species) ($r>0.85$) as well as beech ($r=0.84$). The strongest correlation in IgG₄ titers was seen between birch and alder ($r=0.93$). There was no correlation between birch and chestnut ($r=0.011$).

BAT

Sera from 12 actively treated and 7 placebo patients were analysed for basophil activation by 6 different pollen allergen extracts to address the functionality of the changes in allergen-specific antibodies towards all of these allergens. Data are presented in figure 6. Sera from all patients facilitated activation for alder, birch and hornbeam, 18/19 for hazel, 17/19 for beech and 12/19 for chestnut. The allergen concentrations needed to activate the basophils differed considerably with EC₅₀ (the allergen concentration at which 50% of maximal basophil activation occurs) for all donors below 100 ng for alder and birch whereas 16/19 (hornbeam), 12/18 (hazel), 3/17 (beech), and 4/12 (chestnut) donors had EC₅₀ below 100 ng for the other allergen extracts investigated. Significant increases in median EC₅₀ compared to baseline was observed in the treatment group for birch, alder, and hornbeam, and no significant difference were observed in the placebo group for these tree pollen allergens. Trends for such increases in the treatment group were seen for hazel, beech and chestnut as well in contrast to the placebo group but significant differences were not observed in either group for these allergens.

T-cell cross-reactivity to birch homologous trees in untreated birch allergic patients

An allergic immune response includes both production of allergen-specific IgE and activation of allergen-specific Th2 cells. The T-cell response to allergens from the birch homologous group was investigated in T-cell lines generated from PBMC through stimulation with birch pollen extract. The proliferation data illustrate that T-cell lines respond to allergen extracts from various tree species (Figure 7, left panel) and even though significant variations in the strength of the responses are seen, the majority of patients respond to birch, alder, hazel, and hornbeam (66-100%) whereas beech and chestnut were recognized by 50-60% of the T-cell lines. IL-5 production was observed for all but one of the responses investigated with additional IFN- γ for some T-cell lines (Fig E3, online suppl) indicating primary generation of Th2 responses in these

cultures. T-cell responses to peptide pools covering the entire aa-sequence of the individual major allergens (Figure 7, right panel) show a clear pattern with almost equally frequent responses (90-100%) to group 1 allergens from birch, alder, hazel, and hornbeam and with reduced frequencies of responses to beech and chestnut (70-80%). The strength of the responses to all peptide pools apart from *Aln g 1* peptides differed significantly from the responses to the *Bet v 1* peptides.

Discussion

The current investigation evaluate the sensitization pattern and the antibody responses to birch and multiple birch homologous trees in a cohort of allergic subjects diagnosed with birch pollen allergy. The patients participated in a randomised double-blind, placebo-controlled multicenter phase III trial with the SQ tree SLIT-tablet, which resulted in significant reductions of allergic rhinoconjunctivitis symptoms and medication use during the alder, hazel and birch pollen seasons²⁴. This clinical cross-protection has been debated since the early 1980'es and the tree SLIT-tablet phase III trial provides an unique opportunity to systematically investigate the underlying molecular and cellular mechanisms of immunological cross-reactivity.

Allergic responses in the spring in Europe are almost synonymous with allergy to pollen from birch and other pollinating trees phylogenetically closely related to birch. The sensitization to multiple trees is the result of IgE cross-reactivity as demonstrated by the IgE inhibition and depletion studies^{5,14} leading to the concept of a birch homologous group². This was supported by the strong correlation between serum concentrations of allergen-specific IgE to allergen extracts from trees within the birch homologous group recently observed in more than 200 Canadian birch allergic patients²¹. Similarly, the current data demonstrate that IgE titers towards birch, alder, hazel, hornbeam, and beech are highly correlated in a large cohort of allergic patients (Fig 4, n=397) recruited at multiple sites throughout Europe on basis of IgE and SPT reactivity to birch. Interestingly, highly similar levels of correlation and ranking of the correlations between individual trees were observed when comparing IgE sensitization to multiple birch homologous trees of the European and Canadian²¹ cohorts indicating that this extensive IgE cross-reactivity is commonly observed on both continents. In addition, the ranking of the cross-reactivity based on serum IgE (alder>hazel) was also reflected in skin test with >92% SPT positive for alder^{21,24} and 86% positive for hazel²⁴ suggesting that it may be demonstrated by both diagnostic procedures. Another important point is that all subjects included in these two studies have the strongest response to birch, i.e. none of the subjects with low birch IgE have a higher IgE titer towards any of the other trees, suggesting that birch pollen is the main problem. It is not known whether this is

a result of higher concentrations of major allergen in birch pollens, wide dispersion of birch pollen, or higher pollen counts for birch, but it is seen in multiple studies^{5,14,30,31} and even in areas with low exposure to birch pollens³². Previous reports on T-cell cross-reactivity towards the birch homology group allergens, investigated through the reactivity of Bet v 1-specific T-cell clones or lines, demonstrates cross-reactivity to allergens and peptide epitopes from these related trees as well^{15,17}. In accordance with this, we observed that T-cell reactivity in birch allergic patients is directed towards various birch homologous tree species, which is the result of simultaneous responses to group 1 major allergens from the individual trees (fig 7). Interestingly, T-cell cross-reactivity seemed to be more sensitive to differences in amino acid sequences (i.e. species homology), with 90-100% of patients showing strong proliferation when stimulated with the highly homologous major allergens Bet v 1, Aln g 1, Cor a 1 and Car b 1 and less than 80% of the patients responding with intermediate strength to the allergens Fag s 1, and Cas s 1 with less sequence homology to Bet v 1.

Overall, both IgE and T-cell responses in European birch allergic patients appeared consistently cross-reactive to species of the birch homologous group suggesting that the same IgE antibodies (replicated in Canadian patients) and the same T-cells are causing symptoms when patients are exposed to pollens from any of the birch homologous group trees. Regarding diagnosis, IgE and SPT data demonstrates that a positive test for birch means that alder and hazel (SPT and IgE) as well as hornbeam and beech (IgE) will be positive in more than 80% of these patients. Thus additional tests will not be needed for the majority of the patients.

Although the full mode of action of AIT remains to be understood, it is generally accepted that it needs to address both allergen-specific T- and B-cells in order to be clinically effective²³. Thus, the cross reactive allergen-specific IgE and T-cell responses characteristic for a tree pollen allergic immune phenotype are targets of AIT across various birch homologous trees. The clinical effect of SQ tree SLIT-tablets demonstrated for the birch pollen season²⁴ was accompanied by changes in Bet v-specific IgE (Fig 1), IgG₄ (Fig 2), and IgE-BF (Fig 3) with kinetics very similar to the changes seen for SLIT-tablet treatment of grass, ragweed, and mite allergy^{33,34,35} indicating a common mode of action for this type of AIT. The current study design do not allow for an investigation of the long-term effect of the tree SLIT-tablets. However, a lasting treatments effect has been observed in

grass SLIT-tablet studies for adults as well as children and recently also for japanese cedar SLIT-tablets³⁶. The immunological changes observed in the current study closely parallels what is observed during the first year of grass SLIT-tablet treatment indicating that a long-term effect

could be expected for the tree SLIT-tablet if treatment is continued in accordance with current recommendations of a 3-year treatment duration.

Interestingly, the changes in birch specific IgG₄ levels were closely paralleled by changes in birch specific IgE-BF, a functional readout for the sum of changes in allergen-specific antibodies (IgE and non-IgE) during AIT. Changes in IgE-BF has previously been demonstrated to correlate with reductions in activation of basophils and reductions in the uptake and presentation of allergens to T-cells in birch AIT^{25,37}. Thus allergen-specific activation of effector cells and of T-cells are expected to be inhibited as well in the current study and this was supported by inhibition of BAT (Fig 6) for stimulation with birch at the end of treatment.

The changes observed for birch were extended upon by analysing changes in IgE, IgG₄, and IgE-BF for alder and hazel to investigate the impact of tree SLIT-tablet treatment on the cross-reactive response to these tree pollens. Kinetics comparable to the changes for birch were seen for alder and hazel regarding the changes in IgE-BF (Fig 3) as well as in IgG₄ serum concentrations (Fig 2). In addition, the kinetics of the changes in IgE specific to these three tree pollens were almost identical (Fig 1). These novel findings suggest that the immune response to each tree is similarly modulated by treatment with the SQ tree SLIT-tablets. The observation that the average levels of IgG₄ against alder and hazel was lower than observed for birch may be caused by differences in assay sensitivity as discussed for hazel in the online repository. Figure E1 (online repository) shows that the serum IgG₄ values towards hazel allergen extract do not include all IgG₄ specific to Cor a 1 and almost identical kinetics and strength of the IgG₄ ImmunoCAP values was obtained for Bet v, Bet v 1, and Cor a 1 (Figure E2, online repository). In addition, a direct link between a certain level of IgG₄ or IgE-BF values and treatment effect has not been established for any allergy, so the main point is that significant induction of IgG₄ in the SQ tree SLIT-tablet treated group compared to placebo towards alder, birch, and hazel were observed from the same treatment time point onwards. The preseasonal treatment period of 16 weeks was based on the symptom relief observed for birch challenge in the EEC trial²¹ and successfully used for this pivotal phase III trial prior to the start of alder/hazel pollen season²⁴. The parallel modulation of the immune response to alder, birch, and hazel allergens supports that the same preseasonal treatment period is applicable to multiple trees and the marked effect observed after as little as 1 month of treatment may suggest that a reduced preseasonal treatment period should be addressed in future studies. The serum inhibitory activity was also observed for basophil activation in a small subset of patients showing that basophil sensitivity for alder was significantly decreased after treatment and a trend towards a decrease was seen for hazel supporting the functional effect of the shift in the balance between IgE and IgG₄. The

induction of cross-reactive IgG₄ was further supported by remarkably tight correlations of serum IgG₄ concentrations (Fig 5) for the majority of patients (>90%) observed for alder ($r=0.93$) and hazel ($r=0.85$) in line with the clinical effect seen in the alder/hazel season²⁴. These correlations are in agreement with the previously reported data from Canadian patients suggesting that also the response to treatment is very similar across these two continents²¹.

Pollen counts for, and allergic responses towards beech and hornbeam are sparsely reported in the literature³⁸ and it was not possible to define pollen seasons for these trees in the phase III clinical trial. The level of IgG₄ induced by SLIT (Fig 5) towards birch, beech and hornbeam correlated strongly indicating cross-reactive responses, and thus supporting that allergic responses to these pollens are modulated by the SQ tree SLIT-tablet treatment. The functional extent of the cross-reactivity was exemplified by BAT inhibition experiments in a small subset of patients, which showed that significantly decreased basophil sensitivity was induced by treatment for hornbeam whereas only a trend towards a reduced BAT was observed for beech. T-cell reactivity towards the major hornbeam allergen, Car b 1, in 26/27 T-cell lines responding to birch extract further supports that immune responses to birch and hornbeam are highly cross-reactive. Linkage between T-cell responses to Bet v 1 and the beech major allergen, Fag s 1, were also seen although with reduced frequency and strength compared to alder/hazel/hornbeam. Thus, cross-reactivity of IgE, T-cells and treatment induced IgG₄ suggests that hornbeam and maybe beech as well should be included in the birch homologous group and that the allergic response is affected by tree SLIT tablet treatment. Theoretically, several other tree species such as red oak, hophornbeam, and chestnut could be included in the birch homologous group³. However, we were not able to demonstrate any significant IgE or IgG₄ cross-reactivity to chestnut in the current study. This suggests that, from an immunological point of view, chestnut should not be included in the birch homologous group even though a trend for decreased basophil sensitivity was observed in BAT after end of treatment and a subset of T-cell lines responding to birch extract and Bet v 1 also reacted to chestnut extract and the PR-10 like major allergen, Cas s 1.

This illustrates that a combination of clinical and immunological readouts for multiple tree species may be used to establish the borders for the extent of immunological cross-reactivity (i.e. for IgE and IgG₄ and maybe also T-cells). Based on this it is possible to hypothesize whether treatment with a representative allergen extract will affect allergic responses to a particular tree pollen and the current data indicate that alder, hazel, beech, and hornbeam are within this border whereas chestnut is not. Regarding clinical practice these findings suggest that for the vast majority of patients allergic to pollens from birch, alder, hazel, , hornbeam and possibly also beech, these allergies can be considered one common disease. In addition, treatment with birch AIT will be

clinically effective throughout these pollen seasons in line with the current guidelines on AIT treatment with a representative species for allergy towards allergen sources belonging to the same homologous group².

The current study may have a bias because all the patients included in the trial were selected on basis of IgE and SPT specific to birch. However, as discussed above birch seem to be the dominant sensitization species for more than 85% of patients allergic to birch homologous trees^{5,14,32,39} making the current data relevant for the vast majority of this patients population. As expected in a clinical study, not all samples were available from all patients at all time points, and some assays were performed on patient subsets due to differences in assay labor intensity but since all subsets were randomly selected for the individual assays there should be no bias in regard to the immune changes observed. However, we can not guarantee that the clinical effect described previously²⁴ is fully reflected in the individual subsets. The choice to exclude the birch homologous group member oak from the analysis may also cause some concern, but we found that clinical and immunological changes observed for this tree species during the clinical trial merits a separate publication (manuscript in preparation). Similarly, a thorough investigation of whether the immune changes observed for this group of tree pollen allergens may be used as biomarkers for clinical effect will also be addressed in a subsequent manuscript combining data from Canadian and European subjects treated with the SQ tree SLIT-tablet.

Taken together, the demonstrated IgE and T-cell cross-reactivity fully supports the concept of the birch homologous group as suggested by Lorenz et al² with birch as the appropriate representative species for diagnosis and AIT. Our data further indicates that beech should be included as an additional homologous group member. The consistency of changes in allergen-specific IgE, IgG₄, and IgE-BF during SQ tree SLIT-tablet treatment aligns with and confirms the clinical effects observed during the birch, alder and hazel pollen seasons and the correlations of end-of-treatment serum IgG₄ values suggest that the clinical effect may even be extended to hornbeam and beech.

Acknowledgements:

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Figure legends

Figure 1: Changes in IgE during tree SLIT-tablet treatment. Data are represented as Least Squares Means (LSM) fold change from baseline of allergen extract specific IgE. P-values (corrected for multiple comparisons) were <0.0001 for differences between active (circles, $n=100$) and placebo (squares, $n=100$) at all time points. Error bars represent the 95% confidence limits of the LSM.

Figure 2: Changes in IgG₄ during tree SLIT-tablet treatment. Data are represented as Least Squares Means (LSM) fold change from baseline of allergen extract specific IgG₄. P-values (corrected for multiple comparisons) were <0.0001 for differences between active (circles, $n=100$) and placebo (Squares, $n=100$) at all time points. Error bars represent the 95% confidence limits of the LSM.

Figure 3: Changes in IgE-BF during tree SLIT-tablet treatment. Data are represented as Least Squares Means (LSM) change from baseline of Aln g, Bet v, and Cor a allergen extract specific IgE-BF. P-values (corrected for multiple comparisons) were <0.0001 for differences between active and placebo at all time points except 1 and 4 month time points for hazel (both $p<0,01$). Error bars represent the 95% confidence limits of the LSM.

Figure 4: Correlation between Bet v serum IgE (x-axis) and serum IgE specific to each of the homologous trees (y-axis) at baseline. Lower level of quantification (LLQ) is 0.7 kUA/L and all values below this value were recoded (RC) to 0.35 kUA/L. Pearson correlation r-values and percent samples above LLQ on both axes are indicated for each plot. All correlations were significant ($p<0.0001$) except for birch/chestnut ($p=0.7326$).

Figure 5: Correlation between Bet v serum IgG₄ (x-axis) and serum IgG₄ specific to each of the homologous trees (y-axis) at end of treatment visit. Lower level of quantification (LLQ) is 0.15 mgA/L and all values below this value were recoded (RC) to 0.075 mgA/L. Pearson correlation r-values and percent samples above LLQ on both axes are indicated for each plot. All correlations were significant ($p<0.0001$) except for birch/chestnut ($p=0.9595$).

Figure 6: BAT was performed with passively sensitized basophils, sensitized with pre-IT serum from the individual patients and activated by allergen preparations ranging from 0,25 ng/ml to 250 µg/ml in the presence of pre- or post-IT sera (10%). Changes in EC₅₀ are depicted for Tree SLIT-tablet treated (active, $n=12$) and placebo ($n=7$) treated patients. Significance levels for wilcoxon ranked sum test are indicated for the two treatment groups stimulated with each tree pollen allergen extract. NS: non significant; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

Figure 7: T-cell proliferation in response to allergen extracts (left, $n=29$) and peptide pools (right, $n=27$). Relative magnitudes are indicated for all T-cell lines with $SI>3$ with responses towards Bet v extract set to 100 and all other data displayed relative to this. Responses with $SI<3$ were set to 0. Violin plots depicts distribution of the data. Lines in plots show medians and quartiles. Bet v=Betula verrucosa (birch), Aln g=Alnus glutinosa (alder), Cor a=Corylus avellana (hazel), Car b=Carpinus betulus (hornbeam), Fag s=Fagus sylvatica (beech), Cas s=Castanea sativa (chestnut).

References

- Accepted Article
- (1) Bousquet, P. J.; Chinn, S.; Janson, C.; Kogevinas, M.; Burney, P.; Jarvis, D. Geographical Variation in the Prevalence of Positive Skin Tests to Environmental Aeroallergens in the European Community Respiratory Health Survey I. *Allergy* **2007**, *62*, 301-309.
 - (2) Lorenz, A. R.; Luttkopf, D.; May, S.; Scheurer, S.; Vieths, S. The Principle of Homologous Groups in Regulatory Affairs of Allergen Products--a Proposal. *Int Arch Allergy Immunol* **2009**, *148*, 1-17.
 - (3) Heath, M. D.; Collis, J.; Batten, T.; Hutchings, J. W.; Swan, N.; Skinner, M. A. Molecular, Proteomic and Immunological Parameters of Allergens Provide Inclusion Criteria for New Candidates Within Established Grass and Tree Homologous Groups. *World Allergy Organ. J* **2015**, *8*, 21.
 - (4) Jarolim, E.; Tejkl, M.; Rohac, M.; Schlerka, G.; Scheiner, O.; Kraft, D.; Breitenbach, M.; Rumpold, H. Monoclonal Antibodies Against Birch Pollen Allergens: Characterization by Immunoblotting and Use for Single-Step Affinity Purification of the Major Allergen Bet v I. *Int Arch Allergy Appl. Immunol* **1989**, *90*, 54-60.
 - (5) Ipsen, H.; Wihl, J. A.; Petersen, B. N.; Lowenstein, H. Specificity Mapping of Patients IgE Response Towards the Tree Pollen Major Allergens Aln g I, Bet v I and Cor a I. *Clin Exp Allergy* **1992**, *22*, 391-399.
 - (6) Ipsen, H.; Bowadt, H.; Janniche, H.; Nuchel, P. B.; Munch, E. P.; Wihl, J. A.; Lowenstein, H. Immunochemical Characterization of Reference Alder (*Alnus Glutinosa*) and Hazel (*Corylus Avellana*) Pollen Extracts and the Partial Immunochemical Identity Between the Major Allergens of Alder, Birch and Hazel Pollens. *Allergy* **1985**, *40*, 510-518.
 - (7) Valenta, R.; Breiteneder, H.; Petternburger, K.; Breitenbach, M.; Rumpold, H.; Kraft, D.; Scheiner, O. Homology of the Major Birch-Pollen Allergen, Bet v I, With the Major Pollen Allergens of Alder, Hazel, and Hornbeam at the Nucleic Acid Level As Determined by Cross-Hybridization. *J Allergy Clin Immunol* **1991**, *87*, 677-682.
 - (8) Ipsen, H.; Hansen, O. C. The NH₂-Terminal Amino Acid Sequence of the Immunochemically Partial Identical Major Allergens of Alder (*Alnus Glutinosa*) Aln g I, Birch (*Betula Verrucosa*) Bet v I, Hornbeam (*Carpinus Betulus*) Car b I and Oak (*Quercus Alba*) Que a I Pollens. *Mol. Immunol* **1991**, *28*, 1279-1288.

- (9) Hirschwehr, R.; Valenta, R.; Ebner, C.; Ferreira, F.; Sperr, W. R.; Valent, P.; Rohac, M.; Rumpold, H.; Scheiner, O.; Kraft, D. Identification of Common Allergenic Structures in Hazel Pollen and Hazelnuts: a Possible Explanation for Sensitivity to Hazelnuts in Patients Allergic to Tree Pollen. *J Allergy Clin Immunol* **1992**, *90*, 927-936.
- (10) Larsen, J. N.; Stroman, P.; Ipsen, H. PCR Based Cloning and Sequencing of Isogenes Encoding the Tree Pollen Major Allergen Car b I From Carpinus Betulus, Hornbeam. *Mol. Immunol* **1992**, *29*, 703-711.
- (11) Spangfort, M. D.; Mirza, O.; Holm, J.; Larsen, J. N.; Ipsen, H.; Lowenstein, H. The Structure of Major Birch Pollen Allergens--Epitopes, Reactivity and Cross-Reactivity. *Allergy* **1999**, *54 Suppl 50*, 23-26.
- (12) Mirza, O.; Henriksen, A.; Ipsen, H.; Larsen, J. N.; Wissenbach, M.; Spangfort, M. D.; Gajhede, M. Dominant Epitopes and Allergic Cross-Reactivity: Complex Formation Between a Fab Fragment of a Monoclonal Murine IgG Antibody and the Major Allergen From Birch Pollen Bet v 1. *J Immunol* **2000**, *165*, 331-338.
- (13) Matricardi, P. M.; Kleine-Tebbe, J.; Hoffmann, H. J.; Valenta, R.; Hilger, C.; Hofmaier, S.; Aalberse, R. C.; Agache, I.; Asero, R.; Ballmer-Weber, B.; Barber, D.; Beyer, K.; Biedermann, T.; Bilo, M. B.; Blank, S.; Bohle, B.; Bosshard, P. P.; Breiteneder, H.; Brough, H. A.; Caraballo, L.; Caubet, J. C.; Cramer, R.; Davies, J. M.; Douladiris, N.; Ebisawa, M.; Elgenmann, P. A.; Fernandez-Rivas, M.; Ferreira, F.; Gadermaier, G.; Glatz, M.; Hamilton, R. G.; Hawranek, T.; Hellings, P.; Hoffmann-Sommergruber, K.; Jakob, T.; Jappe, U.; Jutel, M.; Kamath, S. D.; Knol, E. F.; Korosec, P.; Kuehn, A.; Lack, G.; Lopata, A. L.; Makela, M.; Morisset, M.; Niederberger, V.; Nowak-Wegrzyn, A. H.; Papadopoulos, N. G.; Pastorello, E. A.; Pauli, G.; Platts-Mills, T.; Posa, D.; Poulsen, L. K.; Raulf, M.; Sastre, J.; Scala, E.; Schmid, J. M.; Schmid-Grendelmeier, P.; van, H. M.; van, R. R.; Vieths, S.; Weber, R.; Wickman, M.; Muraro, A.; Ollert, M. EAACI Molecular Allergology User's Guide. *Pediatr. Allergy Immunol* **2016**, *27 Suppl 23*, 1-250.
- (14) Niederberger, V.; Pauli, G.; Gronlund, H.; Froschl, R.; Rumpold, H.; Kraft, D.; Valenta, R.; Spitzauer, S. Recombinant Birch Pollen Allergens (RBet v 1 and RBet v 2) Contain Most of the IgE Epitopes Present in Birch, Alder, Hornbeam, Hazel, and Oak Pollen: a Quantitative IgE Inhibition Study With Sera From Different Populations. *J Allergy Clin Immunol* **1998**, *102*, 579-591.

- (15) Ebner, C.; Ferreira, F.; Hoffmann, K.; Hirschwehr, R.; Schenk, S.; Szepefalusi, Z.; Breiteneder, H.; Parronchi, P.; Romagnani, S.; Scheiner, O.; . T Cell Clones Specific for Bet v I, the Major Birch Pollen Allergen, Crossreact With the Major Allergens of Hazel, Cor a I, and Alder, Aln g I. *Mol. Immunol* **1993**, *30*, 1323-1329.
- (16) Schenk, S.; Hoffmann-Sommergruber, K.; Breiteneder, H.; Ferreira, F.; Fischer, G.; Scheiner, O.; Kraft, D.; Ebner, C. Four Recombinant Isoforms of Cor a 1, the Major Allergen of Hazel Pollen, Show Different Reactivities With Allergen-Specific T-Lymphocyte Clones. *Eur J Biochem.* **1994**, *224*, 717-722.
- (17) Sparholt, S. H.; Larsen, J. N.; Ipsen, H.; Schou, C.; van Neerven, R. J. Crossreactivity and T-Cell Epitope Specificity of Bet v 1-Specific T Cells Suggest the Involvement of Multiple Isoallergens in Sensitization to Birch Pollen. *Clin Exp Allergy* **1997**, *27*, 932-941.
- (18) Bohle, B.; Radakovics, A.; Luttkopf, D.; Jahn-Schmid, B.; Vieths, S.; Ebner, C. Characterization of the T Cell Response to the Major Hazelnut Allergen, Cor a 1.04: Evidence for a Relevant T Cell Epitope Not Cross-Reactive With Homologous Pollen Allergens. *Clin Exp Allergy* **2005**, *35*, 1392-1399.
- (19) EMA. EMA guideline on allergen products: production and quality issues (EMA/CHMP/BWP/304831/2007). 2007.

Ref Type: Generic

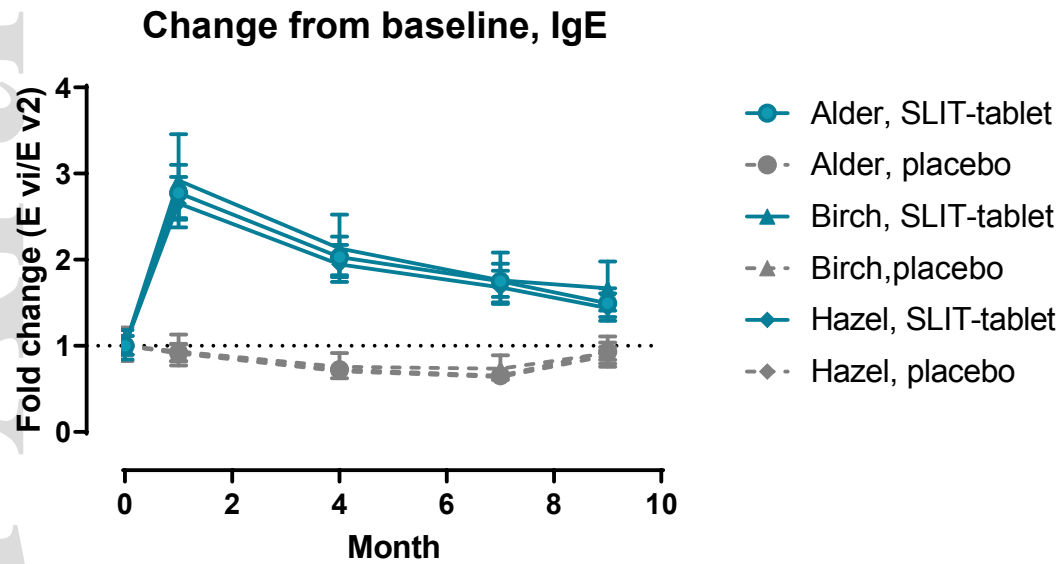
- (20) Cox, L.; Nelson, H.; Lockey, R.; Calabria, C.; Chacko, T.; Finegold, I.; Nelson, M.; Weber, R.; Bernstein, D. I.; Blessing-Moore, J.; Khan, D. A.; Lang, D. M.; Nicklas, R. A.; Oppenheimer, J.; Portnoy, J. M.; Randolph, C.; Schuller, D. E.; Spector, S. L.; Tilles, S.; Wallace, D. Allergen Immunotherapy: a Practice Parameter Third Update. *J Allergy Clin Immunol* **2011**, *127*, S1-S55.
- (21) Couroux, P.; Ipsen, H.; Stage, B. S.; Damkjaer, J. T.; Steffensen, M. A.; Salapatek, A. M.; Lund, K.; Wurtzen, P. A. A Birch Sublingual Allergy Immunotherapy Tablet Reduces Rhinoconjunctivitis Symptoms When Exposed to Birch and Oak and Induces IgG₄ to Allergens From All Trees in the Birch Homologous Group. *Allergy* **2018**.
- (22) Mothes, N.; Horak, F.; Valenta, R. Transition From a Botanical to a Molecular Classification in Tree Pollen Allergy: Implications for Diagnosis and Therapy. *Int Arch Allergy Immunol* **2004**, *135*, 357-373.

- (23) Shamji, M. H.; Durham, S. R. Mechanisms of Allergen Immunotherapy for Inhaled Allergens and Predictive Biomarkers. *J Allergy Clin Immunol* **2017**, *140*, 1485-1498.
- (24) Biedermann, T.; Kuna, P.; Panzner, P.; Valovirta, E.; Andersson, M.; de, B. F.; Thrane, D.; Jacobsen, S. H.; Stage, B. S.; Winther, L. Sublingual Immunotherapy With the SQ Tree SLIT-Tablet Is Highly Effective and Well Tolerated: Results From a Randomized, Double-Blind, Placebo-Controlled Phase III Trial. *J Allergy Clin Immunol* **2019**.
- (25) Wurtzen, P. A.; Lund, G.; Lund, K.; Arvidsson, M.; Rak, S.; Ipsen, H. A Double-Blind Placebo-Controlled Birch Allergy Vaccination Study II: Correlation Between Inhibition of IgE Binding, Histamine Release and Facilitated Allergen Presentation. *Clin Exp Allergy* **2008**, *38*, 1290-1301.
- (26) Kleine, B., I; de Heer, P. G.; van der Zee, J. S.; Aalberse, R. C. The Stripped Basophil Histamine Release Bioassay As a Tool for the Detection of Allergen-Specific IgE in Serum. *Int. Arch. Allergy Immunol.* **2001**, *126*, 277-285.
- (27) Lund, G.; Brand, S.; Ramos, T.; Jimeno, L.; Boissy, P.; Vega, F.; Arina, M.; Christensen, L. H.; Hoof, I.; Meno, K. H.; Barber, D.; Blanco, C.; Wurtzen, P. A.; Andersen, P. S. Strong and Frequent T-Cell Responses to the Minor Allergen Phl p 12 in Spanish Patients IgE-Sensitized to Profilins. *Allergy* **2018**, *73*, 1013-1021.
- (28) Gajhede, M.; Osmark, P.; Poulsen, F. M.; Ipsen, H.; Larsen, J. N.; Joost van Neerven, R. J.; Schou, C.; Lowenstein, H.; Spangfort, M. D. X-Ray and NMR Structure of Bet v 1, the Origin of Birch Pollen Allergy. *Nat. Struct. Biol.* **1996**, *3*, 1040-1045.
- (29) Wallner, M.; Erler, A.; Hauser, M.; Klinglmayr, E.; Gadermaier, G.; Vogel, L.; Mari, A.; Bohle, B.; Briza, P.; Ferreira, F. Immunologic Characterization of Isoforms of Car b 1 and Que a 1, the Major Hornbeam and Oak Pollen Allergens. *Allergy* **2009**, *64*, 452-460.
- (30) Hauser, M.; Asam, C.; Himly, M.; Palazzo, P.; Voltolini, S.; Montanari, C.; Briza, P.; Bernardi, M. L.; Mari, A.; Ferreira, F.; Wallner, M. Bet v 1-Like Pollen Allergens of Multiple Fagales Species Can Sensitize Atopic Individuals. *Clin Exp Allergy* **2011**, *41*, 1804-1814.
- (31) Moverare, R.; Westritschnig, K.; Svensson, M.; Hayek, B.; Bende, M.; Pauli, G.; Sorva, R.; Haahtela, T.; Valenta, R.; Elfman, L. Different IgE Reactivity Profiles in Birch Pollen-Sensitive Patients From

Six European Populations Revealed by Recombinant Allergens: an Imprint of Local Sensitization. *Int Arch Allergy Immunol* **2002**, *128*, 325-335.

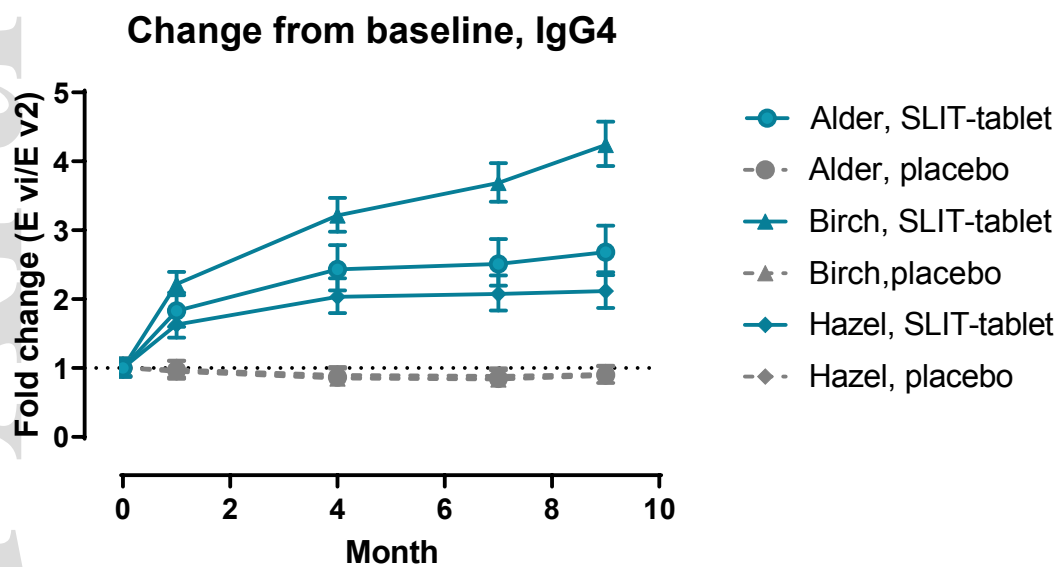
- (32) Mari, A.; Wallner, M.; Ferreira, F. Fagales Pollen Sensitization in a Birch-Free Area: a Respiratory Cohort Survey Using Fagales Pollen Extracts and Birch Recombinant Allergens (RBet v 1, RBet v 2, RBet v 4). *Clin Exp Allergy* **2003**, *33*, 1419-1428.
- (33) Durham, S. R.; Emminger, W.; Kapp, A.; de Monchy, J. G.; Rak, S.; Scadding, G. K.; Wurtzen, P. A.; Andersen, J. S.; Tholstrup, B.; Riis, B.; Dahl, R. SQ-Standardized Sublingual Grass Immunotherapy: Confirmation of Disease Modification 2 Years After 3 Years of Treatment in a Randomized Trial. *J Allergy Clin Immunol* **2012**, *129*, 717-725.
- (34) Creticos, P. S.; Maloney, J.; Bernstein, D. I.; Casale, T.; Kaur, A.; Fisher, R.; Liu, N.; Murphy, K.; Nekam, K.; Nolte, H. Randomized Controlled Trial of a Ragweed Allergy Immunotherapy Tablet in North American and European Adults. *J Allergy Clin Immunol* **2013**, *131*, 1342-1349.
- (35) Virchow, J. C.; Backer, V.; Kuna, P.; Prieto, L.; Nolte, H.; Villesen, H. H.; Ljorring, C.; Riis, B.; de, B. F. Efficacy of a House Dust Mite Sublingual Allergen Immunotherapy Tablet in Adults With Allergic Asthma: A Randomized Clinical Trial. *JAMA* **2016**, *315*, 1715-1725.
- (36) Gotoh, M.; Yonekura, S.; Imai, T.; Kaneko, S.; Horikawa, E.; Konno, A.; Okamoto, Y.; Okubo, K. Long-Term Efficacy and Dose-Finding Trial of Japanese Cedar Pollen Sublingual Immunotherapy Tablet. *J Allergy Clin Immunol Pract.* **2019**, *7*, 1287-1297.
- (37) van Neerven, R. J.; Arvidsson, M.; Ipsen, H.; Sparholt, S. H.; Rak, S.; Wurtzen, P. A. A Double-Blind, Placebo-Controlled Birch Allergy Vaccination Study: Inhibition of CD23-Mediated Serum-Immunoglobulin E-Facilitated Allergen Presentation. *Clin Exp Allergy* **2004**, *34*, 420-428.
- (38) Egger, C.; Focke, M.; Bircher, A. J.; Scherer, K.; Mothes-Luksch, N.; Horak, F.; Valenta, R. The Allergen Profile of Beech and Oak Pollen. *Clin Exp Allergy* **2008**, *38*, 1688-1696.
- (39) Kleine-Tebbe, J.; Zuberbier, T.; Werfel, T.; Krull, M.; Wagenmann, M.; Johansen, N.; Adler, W. P.; Wolf, H.; Mucke, V.; Wustenberg, E.; Biedermann, T. Is Allergy Immunotherapy With Birch Sufficient to Treat Patients Allergic to Pollen of Tree Species of the Birch Homologous Group? *Allergy* **2019**.

1 Figure 1: Changes in IgE during tree SLIT-tablet treatment.
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7 Figure 2: Changes in IgG4 during tree SLIT-tablet treatment.

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Figure 3: Changes in IgE-BF during tree SLIT-tablet treatment.

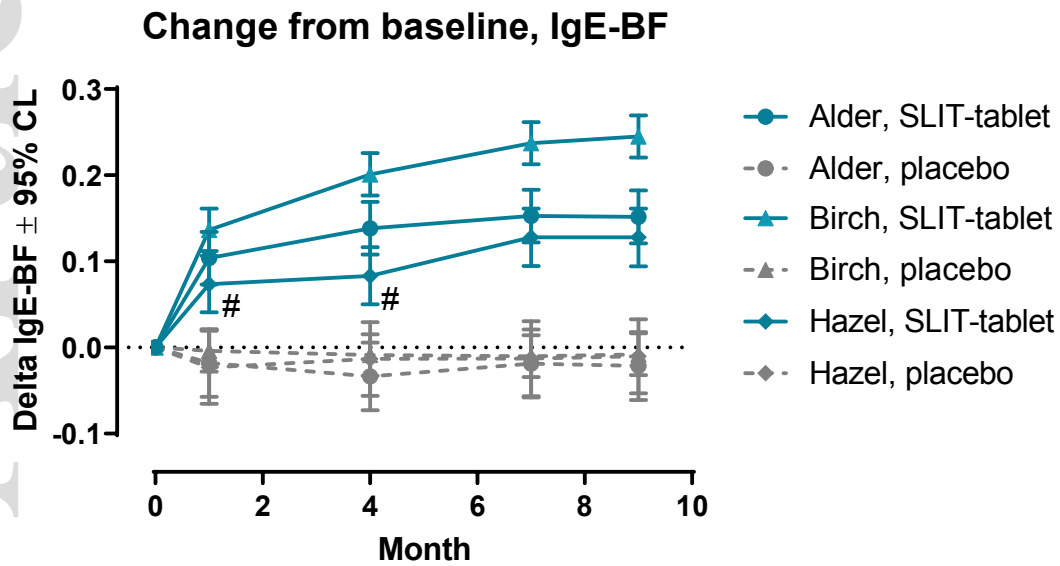


Figure 4: Correlation between birch IgE and homologous species IgE

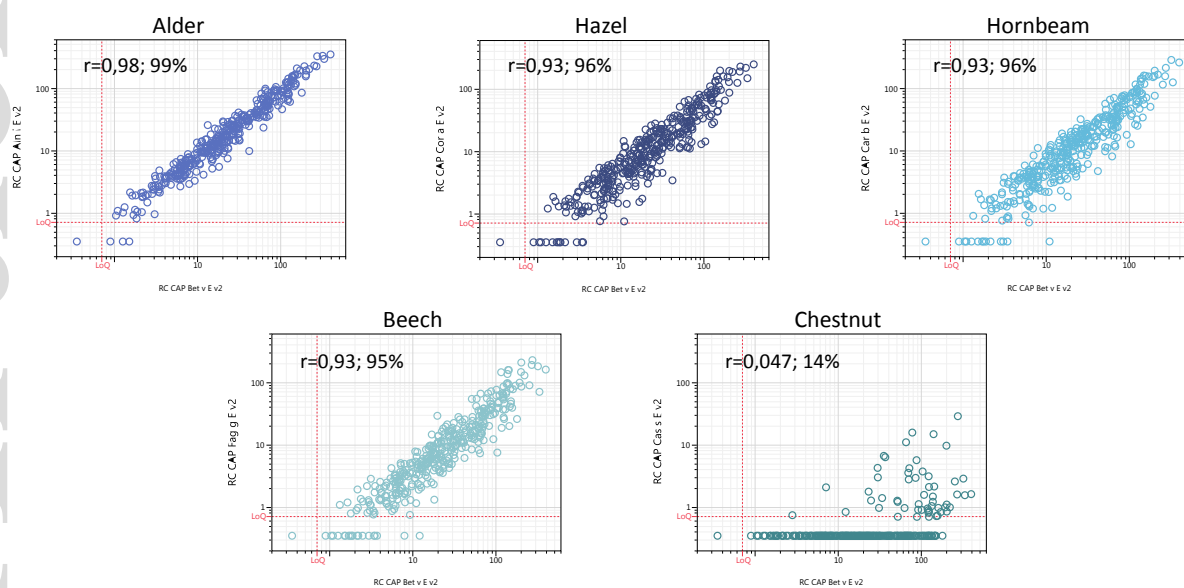
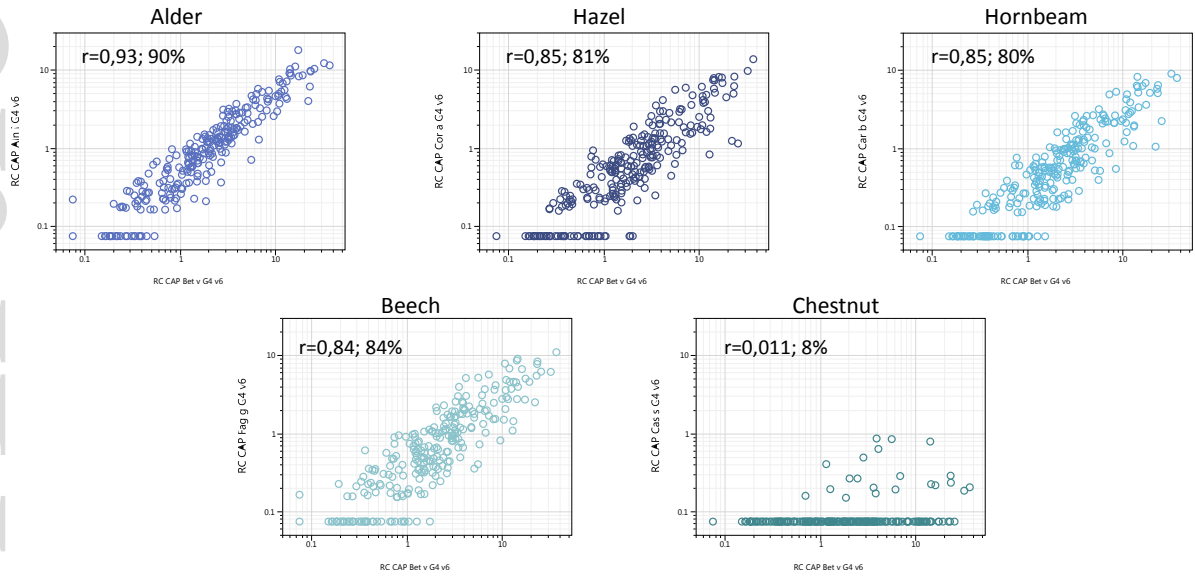


Figure 5: Correlation between birch IgG₄ and homologous species IgG₄



23 Figure 6: Inhibition of Basophil activation induced by tree SLIT-tablet treatment

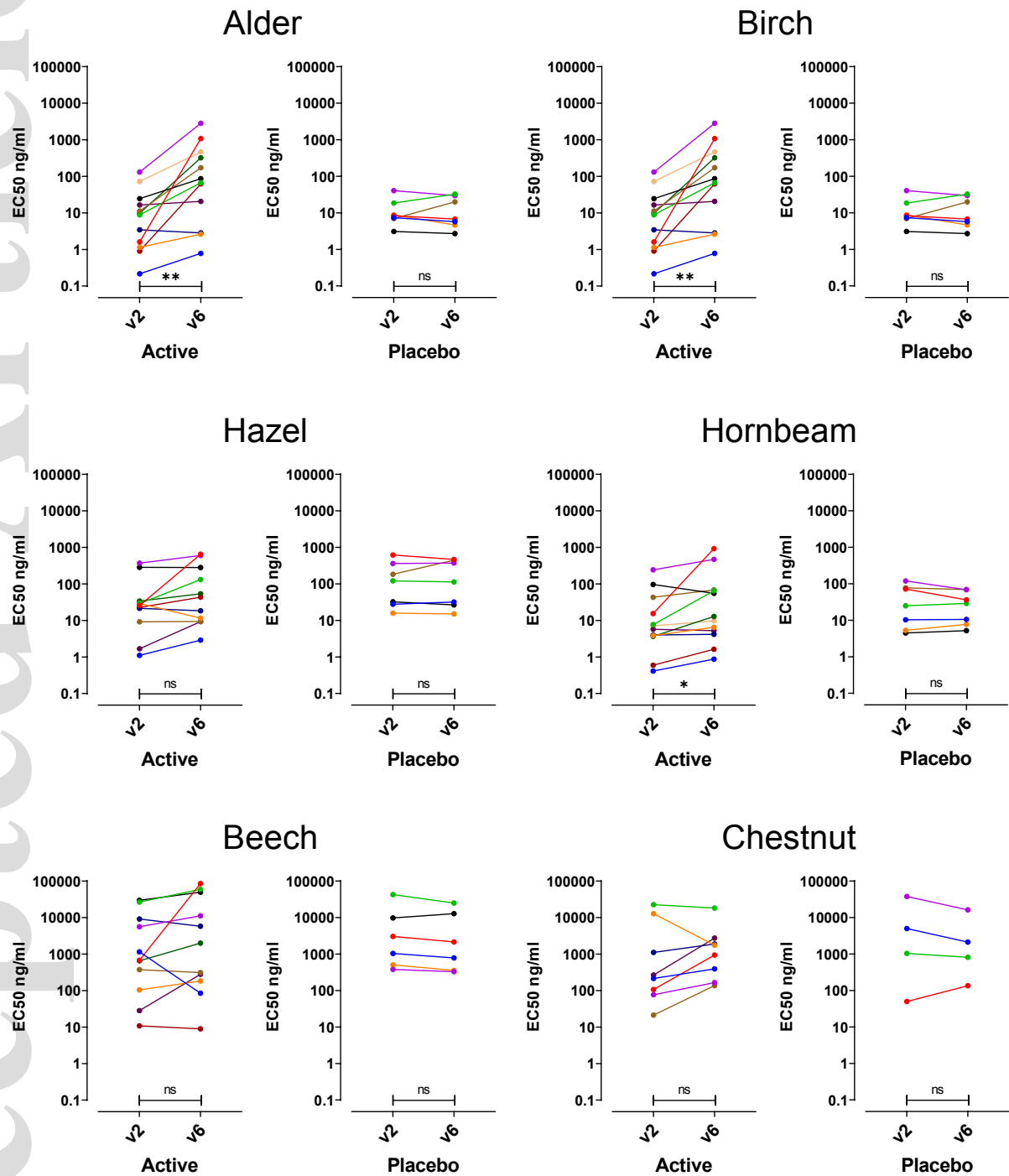
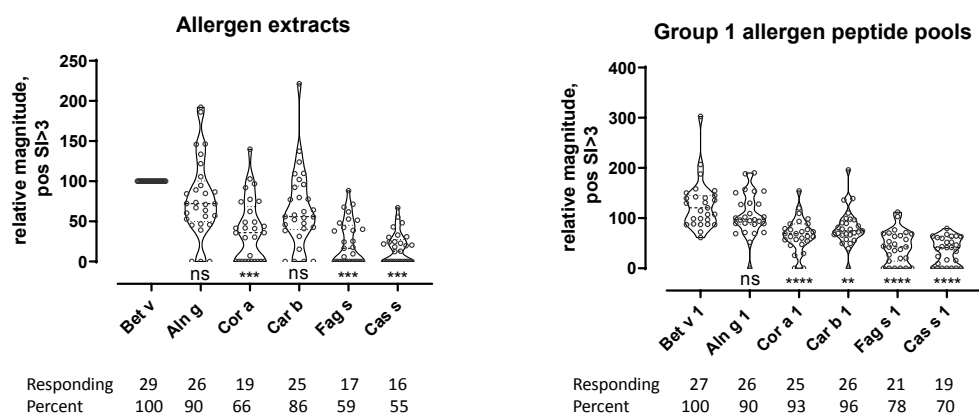


Figure 7: T-cell responses to tree pollen extracts and group 1 allergen peptides



Simplified AIT for allergy to several tree pollens - Arguments from the immune outcome analyses following treatment with SQ tree SLIT-tablet

Online supplement

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Online supplement

Performance of the Cor a IgG4 ImmunoCAP assay

The average level of serum IgG4 induced by tree tablet treatment differs somewhat when measured by the ImmunoCAP using birch, alder, and hazel allergen extracts even though this difference is less pronounced for serum IgE concentrations. The performance of the IgG4 assay for hazel extract may be evaluated to some extent by comparing serum IgG4 concentrations to hazel extract with concentrations to hazel major allergen Cor a 1 because both of these assays are available for the ImmunoCAP assay. In addition, the IgG4 responses towards major allergens Bet v 1 and Cor a 1 and towards the corresponding tree pollen extracts Bet v and Cor a were compared to further understand the differences observed.

Methods

28 50 actively treated patients from the phase III trial (ref) were selected at random for evaluation of
29 the method used for IgG4 measurements.
30 Serum samples collected at baseline and after 1, 4, 7, and 9 month treatment from this patient
31 subset were analysed for Bet v 1 and Cor a 1 serum IgG4 antibodies by ImmunoCAP (Phadia 250,
32 Thermo Fischer Scientific, Uppsala, Sweden).
33 The data were compared to previously obtained data on serum IgG4 antibodies towards the
34 allergen extracts Bet v and Cor a also measured by ImmunoCAP.
35 The change from baseline during treatment was assessed by fitting to a linear mixed model similar
36 to the statistical method for IgE and IgG4 towards the allergen extracts described in the main
37 manuscript.

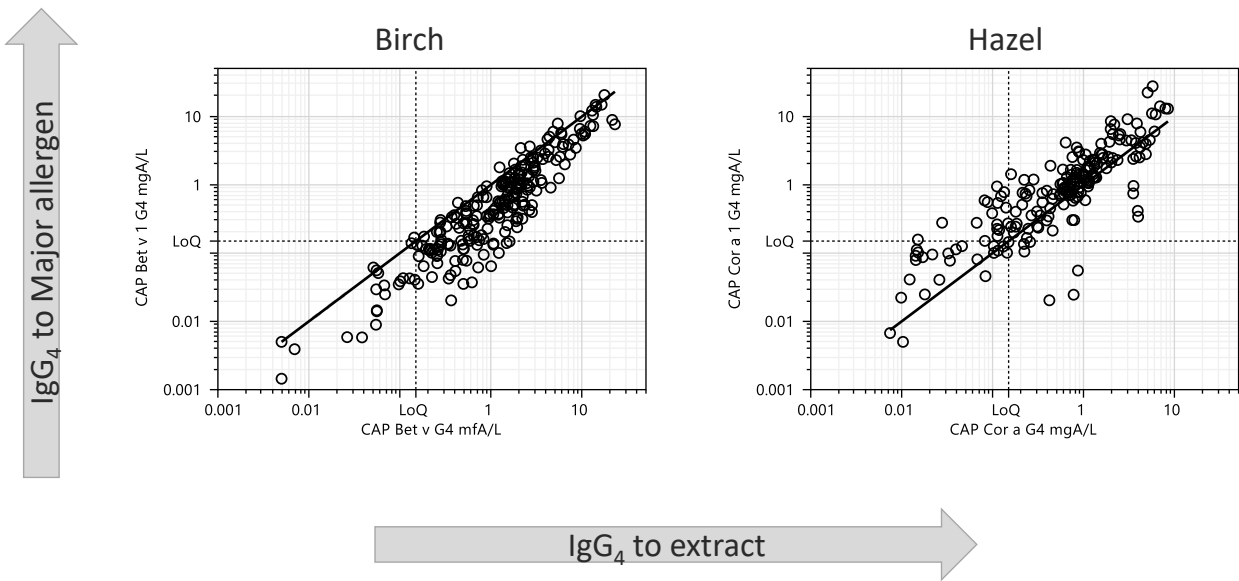
38

39 **Results and discussion**

40 As seen from figure E1, Bet v 1 IgG4 serum concentrations are lower than Bet v IgG4 (right-shift in
41 the scatter-plot), which would be expected considering that Bet v 1 is an individual protein among
42 the multiple allergen/protein components present in an allergen extract. In contrast serum IgG4
43 concentrations to Cor a 1 are higher than to the corresponding hazel extract Cor a (left-shift in the
44 scatter-plot). This is surprising and suggests that a part of the Cor a 1-specific IgG4 is not detected
45 in the Cor a IgG4 assay and that the latter seem to underestimate the sum of hazel component
46 specific IgG4 in the serum samples. Furthermore, if only a part of the total hazel-specific IgG4 is
47 detected by the Cor a IgG4 assay it may be the reason why lower average serum IgG4
48 concentration towards hazel are measured during SQ tree SLIT-tablet treatment than the
49 concentrations measured for birch.

50

Online Figure 1E, IgG₄ correlations extract vs. major allergen



51

52

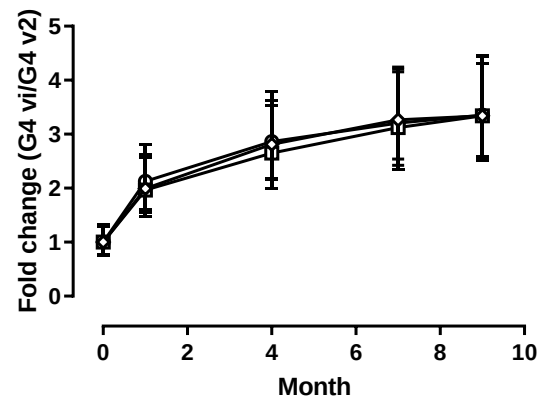
53 Scatter-plots of Bet v or Cor a serum IgG₄ (x-axis) and serum IgG₄ specific to either Bet v 1 or Cor a 1 (y-axis). Lower
54 level of quantification (LLQ) is indicated by dashed lines (0.15 mgA/L) for both axes. Solid lines indicate line of identity
55 (x=y).

56

57 This was further investigated by comparing the Least Squares Means modelled kinetics of IgG₄
58 towards Bet v 1 and Cor a 1 with the kinetics for birch extracts (Bet v) where data were available
59 for all 50 patients (figure E2). The data clearly shows that the average change from baseline in Bet
60 v, Bet v 1 and Cor a 1-specific IgG₄ are almost identical throughout the treatment duration with a
61 maximum fold change above three after 7 and 9 month treatment. This further supports the notion
62 that the only 2-fold increases seen towards hazel extract (figure 2, main manuscript) would have
63 been higher if all the Cor a 1-specific IgG₄ was detected by the Cor a assay.

64

Online Figure E2: Fold change of Bet v 1, Cor a 1 and Bet v IgG₄ during treatment



65

66 Data are represented as Least Squares Means (LSM) fold change from baseline of allergen extract specific IgG₄ (mean

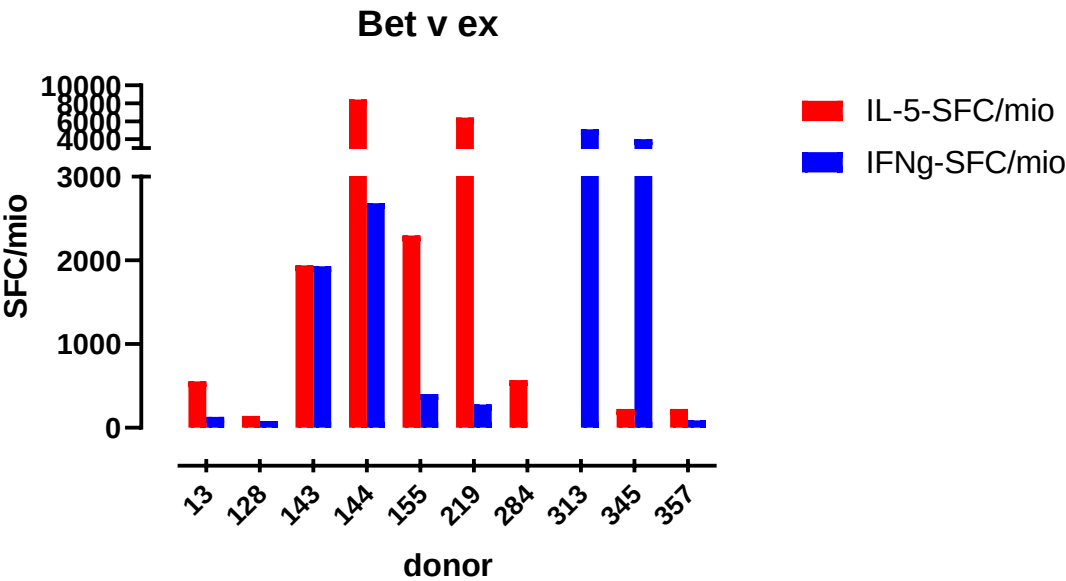
67 +/- 95% conf). Bet v=Betula verrucosa/birch (circles), Bet v 1=birch group 1 major allergen (diamonds) Cor a 1=hazel

68 group 1 major allergen (squares).

69

70 T-cell production of cytokines in response to Bet v

71 Online fig E3: Predominant generation of Th2 T-cell lines



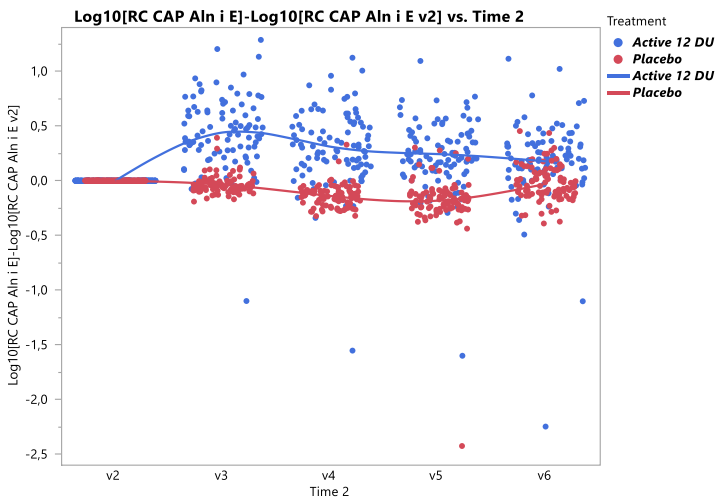
73 T-cell cytokine production was evaluated by fluoroSPOT analysis 10 T-cell lines with sufficient cell
74 numbers. Cells were isolated at day 14 after initial stimulation with Bet v and the number of cells
75 producing IL-5 or IFN-g (per mio cells) on day 3 after restimulation with Bet v are depicted with
76 backgrounds subtracted. 9/10 T-cell lines had cells produced IL-5, 7/10 mainly contained IL-5
77 producing cells, 1/10 equal numbers of IL-5 and IFN-g producing cells, and two cell lines were
78 dominated by/exclusively contained IFN-g producing cells. Cytokine producing cells per mio cells
79 varied from around 100-6000 for IFN-g and around 200-8000 for IL-5.

80

81 **Additional online figs – data from individual patients**

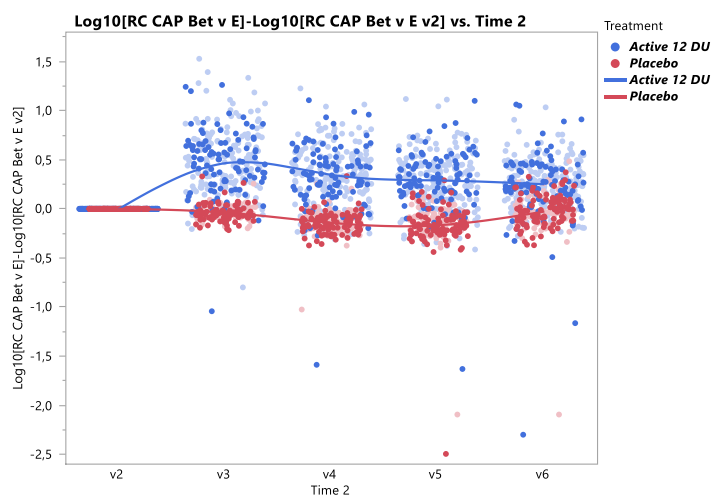
82 Online fig E4: Change from baseline in serum IgE

83 A, Alder



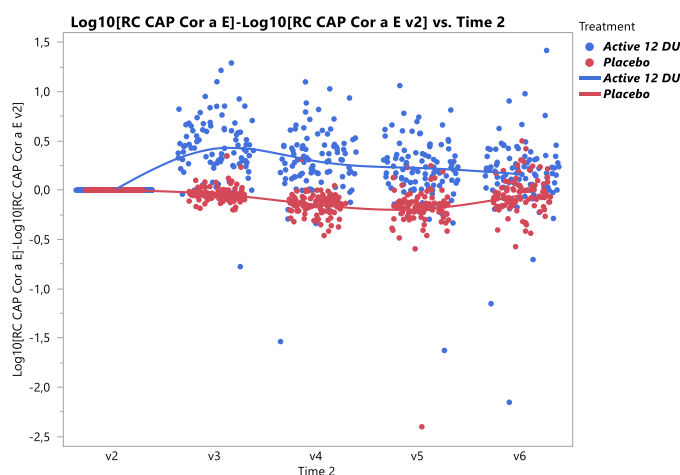
84

85 B, Birch



86

87 C, Hazel



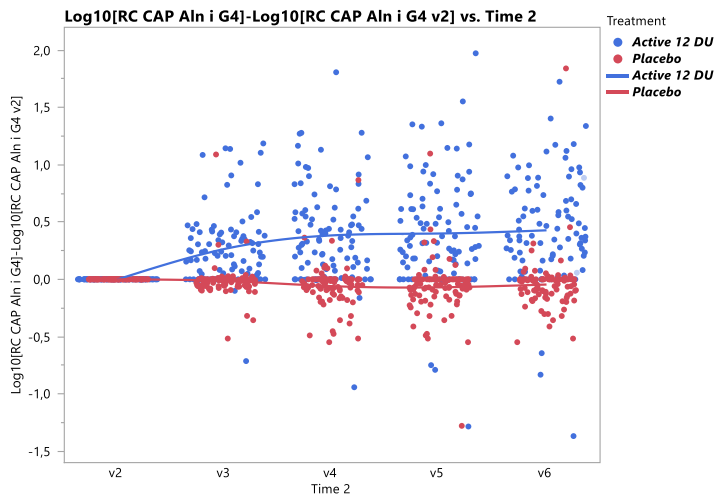
88

89 Changes in IgE antibody responses were investigated as described in the material and method
 90 section of the main manuscript and data for the individual subjects in the active (blue) and the
 91 placebo group (red) are depicted for transparency regarding modelling of the data (fig 1). Dark
 92 blue/red dots for birch-specific IgE measurements are indicating the subset used in the current
 93 investigation matching the subset analysed for alder- and hazel-specific IgE.

94

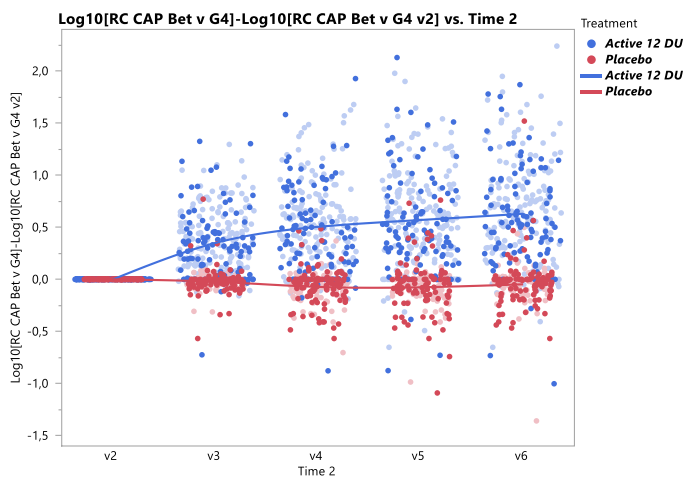
95 Online fig E5: Change from baseline in serum IgG4

96 A, Alder



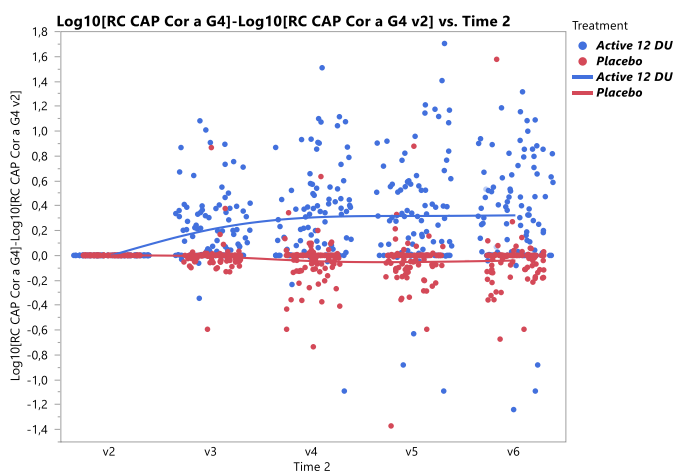
97

98 B, Birch



99

100 C, Hazel



101

102 Changes in IgG4 antibody responses were investigated as described in the material and method
103 section of the main manuscript and data for the individual subjects in the active (blue) and the
104 placebo group (red) are depicted for transparency regarding modelling of the data (fig 2). Dark
105 blue/red dots for birch-specific IgE measurements are indicating the subset used in the current
106 investigation matching the subset analysed for alder- and hazel-specific IgG4.
107