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ORIGINAL ARTICLE

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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

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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 need further investigation. We hypothesize that birch pollen extract AIT modulates 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 IgG_4 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 was 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 towards birch, alder, hazel, hornbeam and beech were observed (*r*-values > .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-IgG_4. IgG_4 reactivity towards birch and alder, hazel, hornbeam and beech correlated significantly at end-of-treatment

Abbreviations: AIT, Allergy immunotherapy; Aln i/g, Alnus incana/glutinosa (alder); BAT, Basophil activation test; Bet v, Betula verrucosa/pendula (birch); Car b, Carpinus betulus (hornbeam); Cas s, Castanea sativa (chestnut); Cor a, Corylus avellana (hazel); EEC, Environmental exposure chamber; FAB, Facilitated allergen binding; Fag g/s, Fagus grandifolia/sylvatica (beech); IgE, IgE; IgE-BF, IgE-blocking factor; IgG_4, IgG_4; LLQ, Lower level of quantification; LME, Linear mixed effects model; MS, Mass spectrometry; PBMC, Peripheral blood mononuclear cells; PHA, Phytohaemagglutinin; PR-10, Pathogenesis-related protein-10; Que a, Quercus alba (oak); RC, Recoded; SI, Stimulation Index; SLIT-tablet, Sublingual allergy immunotherapy tablet; SQ, Standard Quality.

(*r*-values > .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 provide 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.

KEYWORDS

allergy immunotherapy, birch homologous group, cross-reactivity, IgE, IgG_4, immunological mechanism, SLIT-tablet, T cells

1 | 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 worldwide.[1] 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 group[2] termed the 'birch homologous group' and additional trees may be included when more knowledge becomes available.[3] Bet v 1 is the major allergen in birch[4,5] and various birch-related trees contain PR-10 like molecules with high sequence identity to Bet v 1[6-10] and very similar tertiary structures.[11,12] Several minor allergens are also shared between some or all of the birch homologous tree species,[13] 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.[15-18] All of these findings are the foundation for the definition of the birch homologous group of trees.[2] 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 extracts 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 regions.[22]

The changes in the allergen-specific immune response during AIT with allergen extracts include induction of blocking non-IgE antibodies, as well as shifting the balance between Th1/Th2 and regulatory T cells.[23] 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 IgG_4 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 species.[24] 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 IgG_4 towards birch[24] were supplemented by analysing the immunological cross-reactivity of serum IgE and IgG_4 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.

2 | METHODS

2.1 | 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 previously.[24] 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 (≥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 was approved by the local ethics committees approving the trial (EudraCT 2015-004821-15) and informed consent was 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 IgG_4 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 (IgG_4 and other isotypes apart from IgE) for binding to the allergen (n = 160-200).
4. Cross-reactivity of treatment-induced IgG_4 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).

2.2 | 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, IgG_4 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 IgG_4 antibodies by ImmunoCAP (Phadia 250, Thermo Fischer Scientific) according to the manufacturers instructions and Bet v IgE-BF was measured as described previously.[25] 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 IgG_4 (*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 IgG_4 (LLQ = 0.15 mgA/L) were given the value LLQ/2.

2.3 | 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) and CD203c_APC (Biolegend) for identification of basophils and CD63-FITC (BD Bioscience) for measuring activation by FACS analysis. Cells were analysed on Fortessa flow-cytometer, 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.

2.4 | T-cell analyses

PBMC from birch pollen allergic patients (Bet v-specific IgE>0.7 kU/L, analysed by 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 FluoroSPOT[27] on day 14 when cell counts allowed and by thymidine incorporation[27] 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 described[27] on basis of significant positive responses (Student's t test, $P < .05$) 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.

2.5 | 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 (0.125mol/L, pH 8.3) at $5^{O}C$ for 2 hours followed by dialysis (MW cut-off 3.5KD), filtration (0.2 mm) 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 differ 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 was below 20 EU/mg except Cor a and Cas s which were below 100 EU/mg.

2.6 | 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.

2.7 | Statistical methodology

Normality was assessed by visual inspection of normal quantile plots of the data (log10 transformed for IgE and IgG_4 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 (ie 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 IgG_4 cross-reactivity were 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.

3 | RESULTS

3.1 | 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 S4 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 threefold 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.

3.2 | IgG_4 changes during treatment

Induction of allergen-specific IgG_4 is a hallmark of AIT and seen as an indicator of the competing non-IgE antibody response induced by

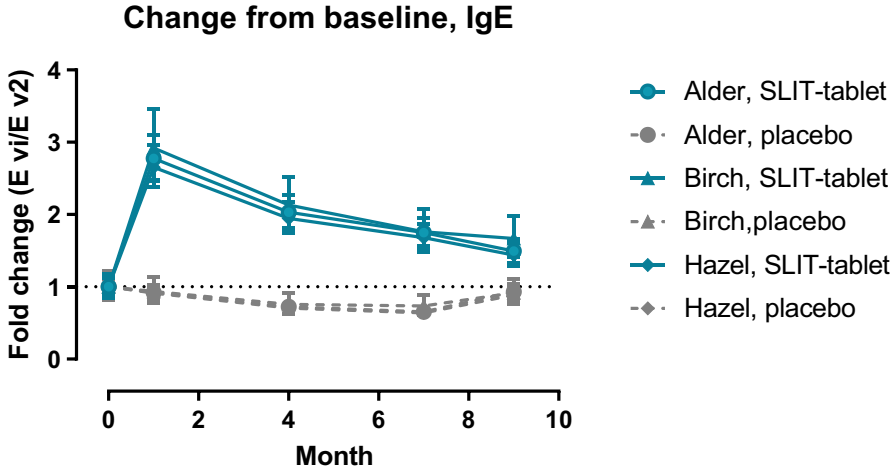

FIGURE 1 Changes in IgE during tree SLIT-tablet treatment. Data are represented as Least Squares Means (LSM) fold change from baseline of allergen extract-specific IgE. *P*-values (corrected for multiple comparisons) were < .0001 for differences between active (circles, n = 100) and placebo (squares, n = 100) at all time points. Error bars represent the 95% confidence limits of the LSM

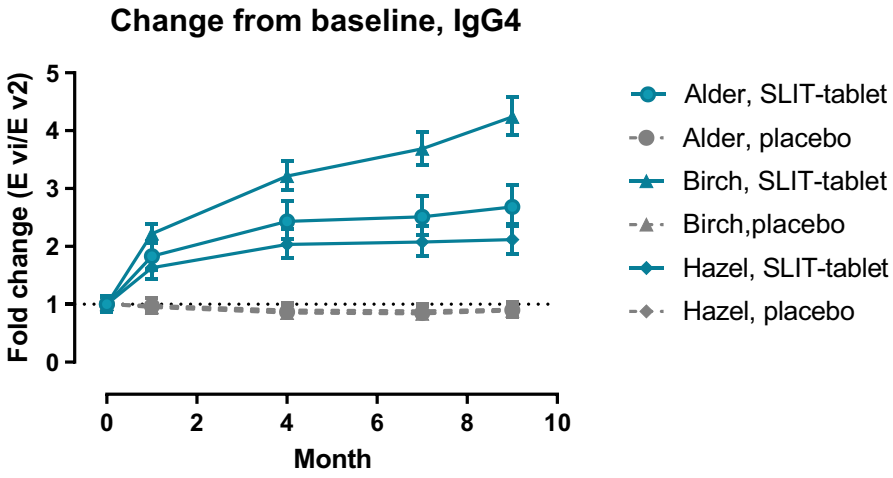

FIGURE 2 Changes in IgG_4 during tree SLIT-tablet treatment. Data are represented as Least Squares Means (LSM) fold change from baseline of allergen extract-specific IgG_4. *P*-values (corrected for multiple comparisons) were < .0001 for differences between active (circles, n = 100) and placebo (Squares, n = 100) at all time points. Error bars represent the 95% confidence limits of the LSM

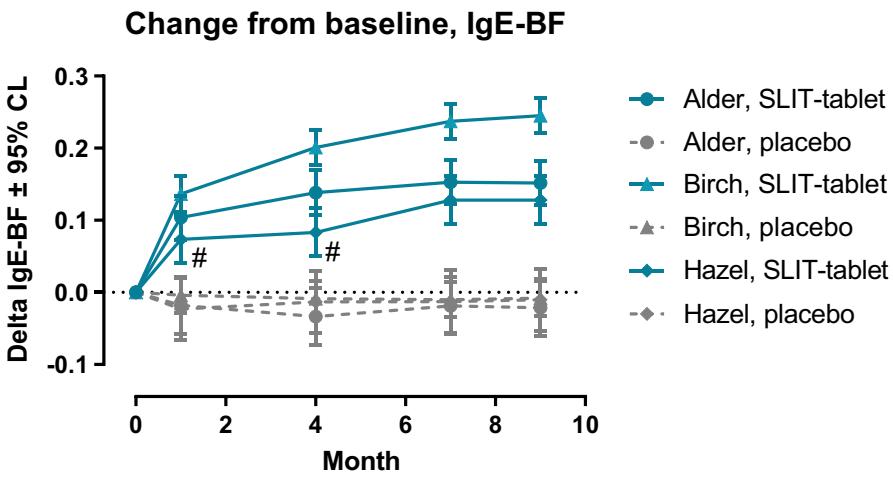

FIGURE 3 Changes in IgE-BF during tree SLIT-tablet treatment. Data are represented as Least Squares Means (LSM) change from baseline of Aln g, Bet v and Cor a allergen extract-specific IgE-BF. *P*-values (corrected for multiple comparisons) were < .0001 for differences between active and placebo at all 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

AIT. A significant induction of allergen-specific IgG_4 was observed in the current trial for alder, birch and hazel after 4 weeks of treatment (Figure 2 and online Figure S5 for data from individual subjects). The serum IgG_4 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.

3.3 | 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 IgG_4. The data depictured 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.

3.4 | 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 sensitization and the correlation between IgE titres towards individual trees of the birch homologous group are 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) ($r \geq .93$) as well as beech ($r = .93$). The strongest correlation in IgE titres was seen between birch and alder ($r = .98$) and the weakest was between birch and hazel or beech ($r = .93$). There was no correlation between birch and chestnut ($r = .047$).

3.5 | Cross-reactivity of end-of-treatment serum IgG_4

Cross-reactivity of IgG_4 indicates that treatment-induced immune modulation affects the response to different closely related tree

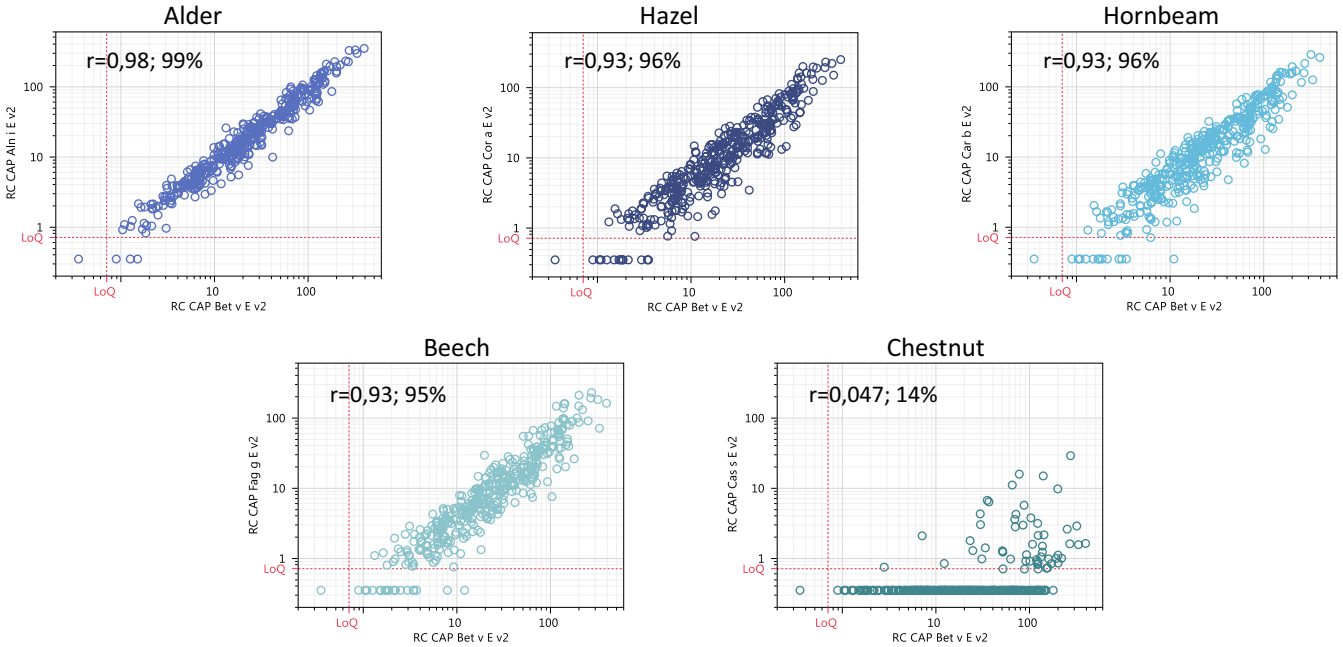

FIGURE 4 Correlation between Bet v serum IgE (x-axis) and serum IgE specific to each of the homologous trees (y-axis) at baseline. Lower level of quantification (LLQ) is 0.7 kUA/L and all values below this value were recoded (RC) to 0.35 kUA/L. Pearson correlation r-values and per cent samples above LLQ on both axes are indicated for each plot. All correlations were significant ($P < .0001$) except for birch/chestnut ($P = .7326$)

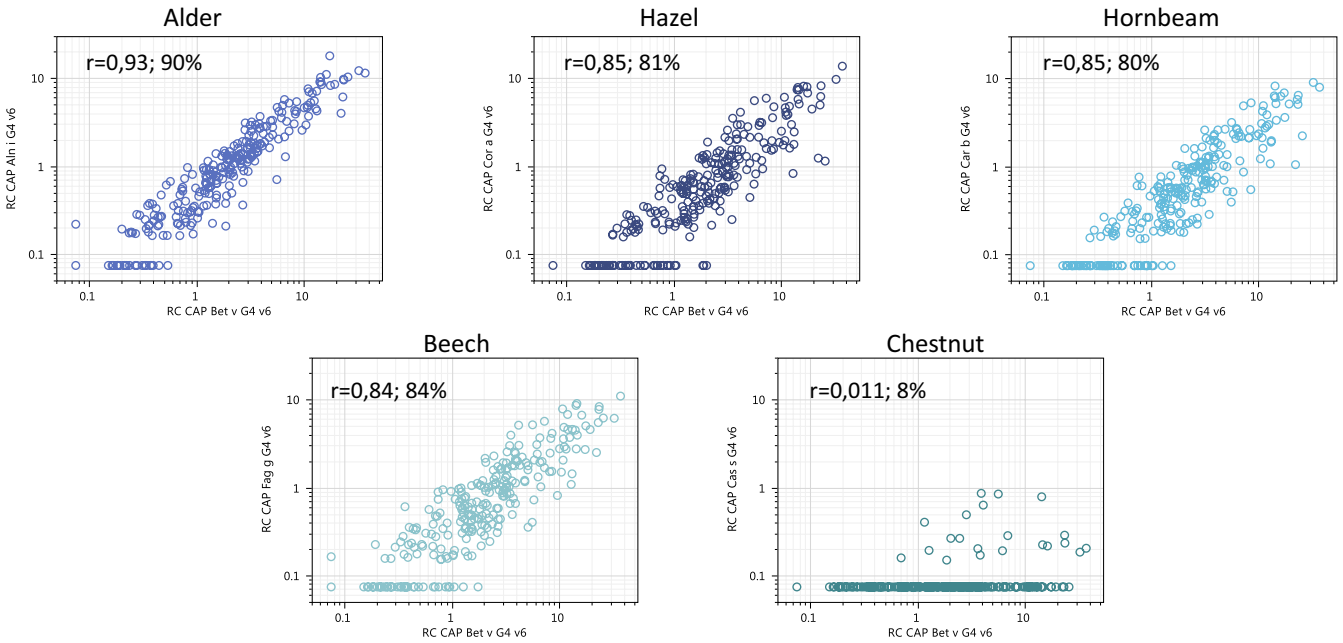

FIGURE 5 Correlation between Bet v serum IgG_4 (x-axis) and serum IgG_4 specific to each of the homologous trees (y-axis) at end-of-treatment visit. Lower level of quantification (LLQ) is 0.15 mgA/L and all values below this value were recoded (RC) to 0.075 mgA/L. Pearson correlation r-values and per cent samples above LLQ on both axes are indicated for each plot. All correlations were significant ($P < .0001$) except for birch/chestnut ($P = .9595$)

species. This was investigated by analysis of correlations similar to the analyses of IgE sensitization. The correlation between IgG_4 serum concentrations towards birch and serum IgG_4 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 IgG_4 binding to multiple related trees ranging from 80% (birch/hornbeam) to 90% (birch/alder), whereas only 8% contained IgG_4 towards chestnut in addition to birch. Significant correlation was found between IgG_4 towards birch and alder, hazel and hornbeam (birch

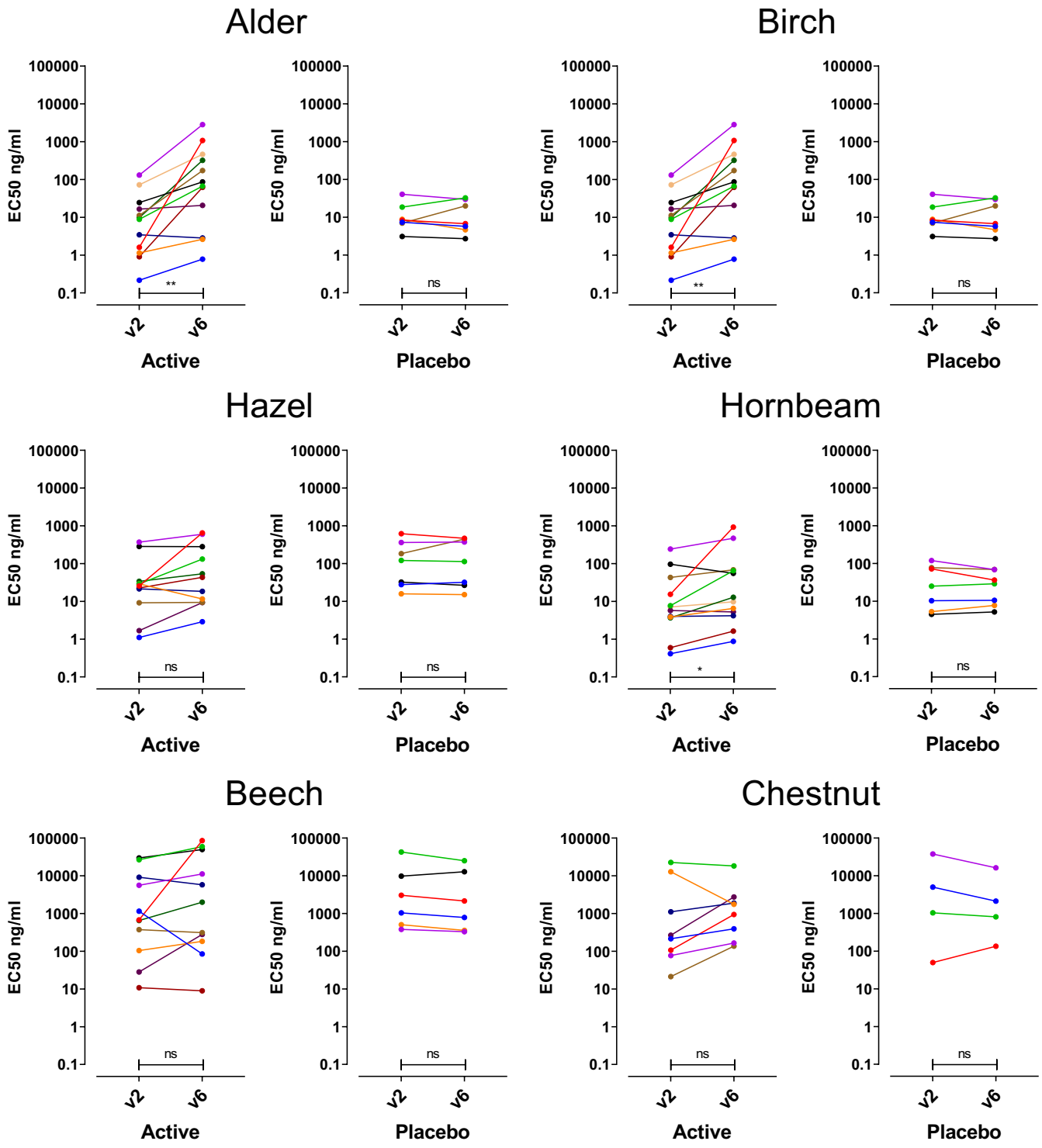

FIGURE 6 BAT was performed with passively sensitized basophils, sensitized with pre-IT serum from the individual patients and activated by allergen preparations ranging from 0,25 ng/ml to 250 µg/ml in the presence of pre- or post-IT sera (10%). Changes in EC50 are depictured for Tree SLIT-tablet-treated (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; *: $P < .05$; **: $P < .01$; ***: $P < .001$

homologous group species) ($r \geq .85$) as well as beech ($r = .84$). The strongest correlation in IgG_4 titres was seen between birch and alder ($r = .93$). There was no correlation between birch and chestnut ($r = .011$).

3.6 | 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 were observed in the treatment group for birch, alder and hornbeam, and no significant difference was 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.

3.7 | 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 (Figure S3, 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.

4 | 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 randomized double-blind, placebo-controlled multicentre 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 seasons.[24] This clinical cross-protection has been debated since the early 1980s 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 group.[2] 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 patients.[21] Similarly, the current data demonstrate that IgE titres towards birch, alder, hazel, hornbeam and beech are highly correlated in a large cohort of allergic patients (Figure 4, n = 397) recruited at multiple sites throughout Europe on basis of IgE and SPT

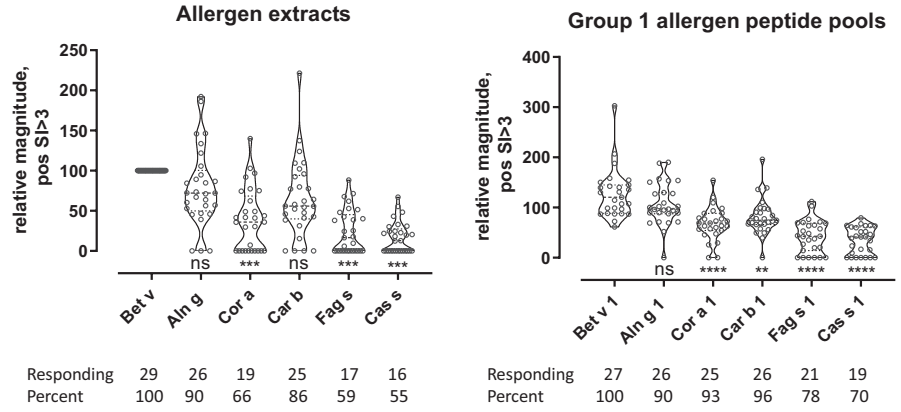

FIGURE 7 T-cell proliferation in response to allergen extracts (left, n = 29) and peptide pools (right, n = 27). Relative magnitudes are indicated for all T-cell lines with SI > 3 with responses towards Bet v extract set to 100 and all other data displayed relative to this. Responses with SI < 3 were 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)

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 Canadian[21] 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 hazel [24] 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, that is, none of the subjects with low birch IgE have a higher IgE titre 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 pollens.[32] 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, demonstrate 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 (Figure 7). Interestingly, T-cell cross-reactivity seemed to be more sensitive to differences in amino acid sequences (ie 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 demonstrate 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 effective.[23] 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 season[24] was accompanied by changes in Bet v-specific IgE (Figure 1), IgG_4 (Figure 2) and IgE-BF (Figure 3) with kinetics very similar to the changes seen for SLIT-tablet treatment of grass, ragweed and mite allergy[33-35] indicating a common mode of action for this type of AIT. The current study design does 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-tablets.[36] 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 IgG_4 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 have 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 is expected to be inhibited as well in the current study and this was supported by inhibition of BAT (Figure 6) for stimulation with birch at the end of treatment.

The changes observed for birch were extended upon by analysing changes in IgE, IgG_4 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 (Figure 3) as well as in IgG_4 serum concentrations (Figure 2). In addition, the kinetics of the changes in IgE specific to these three tree pollens were almost identical (Figure 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 IgG_4 against alder and hazel were lower than observed for birch may be caused by differences in assay sensitivity as discussed for hazel in the online repository. Figure S1 (online repository) shows that the serum IgG_4 values towards hazel allergen extract do not include all IgG_4 specific to Cor a 1 and almost identical kinetics and strength of the IgG_4 ImmunoCAP values were obtained for Bet v, Bet v 1 and Cor a 1 (Figure S2, online repository). In addition, a direct link between a certain level of IgG_4 or IgE-BF values and treatment effect has not been established for any allergy, so the main point is that significant induction of IgG_4 in the SQ tree SLIT-tablet-treated group compared to placebo towards alder, birch and hazel was 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 trial[21] and successfully used for this pivotal phase III trial prior to the start of alder/hazel pollen season.[24] 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 IgG_4. The induction of cross-reactive IgG_4 was further supported by remarkably tight correlations of serum IgG_4 concentrations (Figure 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 season.[24] 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 continents.[21]

Pollen counts and allergic responses towards beech and hornbeam are sparsely reported in the literature[38] and it was not possible to define pollen seasons for these trees in the phase III clinical trial. The level of IgG_4 induced by SLIT (Figure 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 IgG_4 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 group.[3] However, we were not able to demonstrate any significant IgE or IgG_4 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 (ie for IgE and IgG_4 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 group.[2]

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 labour 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 cannot guarantee that the clinical effect described previously[24] 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 merit 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 al[2] with birch as the appropriate representative species for diagnosis and AIT. Our data further indicate that beech should be included as an additional homologous group member. The consistency of changes in allergen-specific IgE, IgG_4 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 IgG_4 values suggest that the clinical effect may even be extended to hornbeam and beech.

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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.

AUTHOR CONTRIBUTIONS

PAW and HI contributed to study design, analysed and interpreted the data, and drafted the manuscript. PMG, and GL performed

experiments and analysed 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.

ROLE OF SPONSOR

The clinical trial was sponsored by ALK, Hoersholm, Denmark, who assumes overall responsibility for the trial and has been involved in both trial design and conduct. All in vitro analyses have been performed by ALK.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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